Enhancing Aluminium Resistance in Barley through Over-expression of *MATE* Genes

by

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DECLARATION

The thesis contains no material, which has been accepted for the award of any other degree or diploma in any tertiary institution, and to the best of my knowledge, contains no material previously published or written by any other person, except where due reference is made in the text of this thesis.

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2,4-D	2,4-dichlorophenoxyacetic acid
ALMT	aluminium-activated malate transporter
Amp	ampicillin
bp	base pair
BSA	bovine serum albumin
BW26	Bobwhite 26
cDNA	complementary DNA
d	day
ddH_2O	didistilled water
Dicamba	3,6-dichloro-o-anisic acid
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E．Coli	Escherichia coli
EDTA	ethylene dinitrilotetracetic acid
g	gram
GP	Golden Promise
h	hour
IPTG	isopropylthio-β-o-galactopyranoside
Kb	kilo base
L	Litre
MATE	multidrug and toxic compound extrusion
mg	milligram
min	minute
mL	millilitre
mRNA	messenger RNA
ng	nanogram
O.D.	optical density
oligo（dT）	oligodeoxythymidylic acid
PCR	polymerase chain reaction
QTL	quatitative trait loci
RG	root growth
RNA	ribonucleic acid
rpm	rounds per minute
RRG	relative root growth
s	second
SDS	sodium dodecyl sulfate
Tris	tris（hytdroxymethyl）aminomethane
U	unit
μg	microgram
μL	microlitre

Abstract

Acid soils limit crop yields around the world due to nutrient deficiencies and mineral toxicities. Non-adapted plants grown on acid soils typically have shorter and thicker root systems because high concentrations of soluble aluminium (Al^{3+}) inhibit root elongation. This restricts their ability to acquire water and nutrients. An important mechanism of Al^{3+} resistance discovered in many plant species relies on the release of organic anions from roots. The gene controlling this trait are members of two gene families called the aluminium activated malate transporter (*ALMT*) family and multidrug and toxic compound exudation (*MATE*) family. Members of the *ALMT* family encode anion channels which release malate anions from roots while the *MATE*s encode co-transporter proteins which facilitate citrate release from roots. Although barley (*Hordeum vulgare*) is more sensitive to Al^{3+} toxicity than many other cereals including wheat (*Triticum aestivum*), rye (*Secale cereale*) and rice (*Oryza sativa*) significant genotypic variation in resistance does occur. This variation is controlled by citrate efflux from the root apices which is encoded by a *MATE* gene called *HvAACT1*. In this study three *MATE* genes from barley, *Arabidopsis* and sorghum (*Sorghum bicolor*) were transformed into the Al^{3+}-sensitive barley cultivar 'Golden Promise' with a constitutive promoter. These genes include the major Al^{3+}-resistance genes from barley and sorghum (*HvAACT1* and *SbMATE* respectively) and the *Frd3* gene from *Arabidopsis thaliana* which is important for iron nutrition. All three are known to encode transport proteins that facilitate citrate efflux from cells. The resulting transgenic lines were assessed for transgene expression, citrate efflux from root apices, and Al^{3+} resistance in hydroponic solution and acid soil. The control plants included in these experiments were null segregant lines and the parental barley cultivar. The Al^{3+}-resistant barley cultivar Dayton was also included as a positive control.

Barley cultivar "Golden Promise" was transformed separately with the *MATE* genes using the *Agrobacterium* method. Several independent T2 or T3 barley lines homozygous for each transgene were generated as well as null segregant lines. The transgenic lines released significantly more citrate from their root apices than the null controls. Plants expressing the *HvAACT1* and *SbMATE* genes required Al^{3+} in the external solution to activate citrate efflux while plants expressing *Frd3* released

citrate in the presence and absence of Al^{3+}. This is consistent with previous studies showing that HvAACT1 and SbMATE are Al^{3+}-activated proteins. The citrate efflux from the transgenic lines was similar to, or greater than, the efflux detected from cv. Dayton.

Transgenic and control seedlings were grown in an aerated hydroponic culture containing a simple nutrient solution with 0, 1, 2, or 4 μM $AlCl_3$ (pH 4.3). Net root growth was measured after 4 d. Relative root growth (growth in the Al^{3+} solution relative to control solution) was significantly greater in the transgenic lines than the null controls for most Al^{3+} treatments and similar results were obtained for the three *MATE* genes. The Al^{3+} resistance of the transgenic lines was similar to the Al^{3+} resistance of cv. Dayton.

Al^{3+} resistance of the transgenic and control lines was also assessed in short-term soil experiments. The acidic ferrosol was either unamended (pH 4.33 with aluminium being 21% of exchangeable cations) or limed so that pH increased to 5.18 and only 1% of exchangeable cations was aluminium. After 6 d growth the following measurements were made: length of the longest and second-longest roots, total root length, total root weight, shoot weight and distribution of root diameters. In the unamended acid soil root growth of the null lines was inhibited compared to the limed soil and the roots became thicker. Expression of each of the *MATE* genes significantly increased Al^{3+} resistance with relative length of the longest roots (root length in acid compared to limed soil) and relative total root length (total root length in acid compared to limed soil) providing the greatest differences between the transgenic and null lines. The transgenic lines also maintained a greater percentage of thinner roots in the acid soil than the null lines.

These results demonstrate that Al^{3+} resistance in barley can be enhanced by heterologous expression of the *SbMATE* and *Frd3* genes or by over-expression of the endogenous *HvAACT1* gene. Biotechnology provides important options for increasing the Al^{3+} resistance of crop plants which can complement traditional breeding practices. Both strategies will be important for maintaining and even increasing food production on acid soils in the future.

Keywords: HvAACT1, citrate transporter, aluminium tolerance, transgene

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CHAPTER 1 Introduction

1. 1 Acid soils

Acid soils limit crop yields around the world due to nutrient deficiencies and mineral toxicities. Non-adapted plants grown on acid soils typically have smaller root systems because high concentrations of soluble aluminium (Al^{3+}) inhibit root elongation. This restricts their ability to acquire water and nutrients and finally affects crop yields.

1.1.1 What is acid soil?

Soil pH is an important consideration for agriculture production (Kochian *et al.*, 2004, Vonuexkull and Mutert, 1995). Some plants are sensitive to high or low pH, nutrient availability and mineral toxicities are influenced by pH and soil microbial communities are significantly affected by pH (Fierer and Jackson, 2006, Osborne *et al.*, 2011). Acid soils present multiple stresses to plants including proton toxicity, nutrient deficiencies (especially calcium, magnesium and phosphorus) and metal-ion toxicities especially aluminium and manganese. The United States Department of Agriculture classifies acid soils into six levels: *ultra acid* soils (below pH 3.5), *extremely acid* (pH 3.5 to 4.4), *very strongly acid* (pH 4.5 to 5.0), *strongly acid* (pH 5.1 to 5.5), *moderately acid* (pH 5.6 to 6.0) and *slightly acid* (pH 6.1 to 6.5). Soils with pH≤5.5 can adversely affect the production of many major food crops (http://soils.usda.gov/technical/handbook/contents/part618.html).

The major limitation to crop growth in acid soils is soluble aluminium. Although aluminium is the third most abundant element in the earth's crust most of it occurs in mineral forms which are harmless to plants (complexes with oxides and silicates). In acid conditions however these minerals dissolve more readily releasing aluminium into the soil solution. Soluble aluminium hydrolyses to form a range of species the prevalence of which depends on soil pH. When the pH is 4.5 or below, the Al^{3+} species predominates but as pH increases other mononuclear aluminium species are formed including $Al(OH)_2^+$ and $Al(OH)^{2+}$. The insoluble $Al(OH)_3$ (gibbsite) can also form at higher pH. Trivalent aluminium (Al^{3+}) is highly toxic to many plants but uncertainty continues regarding the relative toxicity of the hydroxyaluminium species (Alva *et al.*, 1986, Kinraide, 1997, Noble *et al.*, 1988,

Wright *et al.*, 1987). Al^{3+} is a reactive metal ion that forms complexes with a variety of organic and inorganic ligands including carboxylates, sulphate, and phosphate and many of these compounds are less toxic to plants than free Al^{3+} (Jones, 1998, Kinraide, 1997, Matsumoto, 2000, Takita *et al.*, 1999).

1.1.2 Formation and distribution of acid soils

Acid soils can develop naturally depending on characteristics of the parent rock but human intervention can accelerate the process (Rechcigl and Sparks, 1985, Vanbreemen *et al.*, 1983). Ancient and highly-weathered soils are often acid because the basic cations (calcium, magnesium, sodium and potassium) have been leached down the profile, often with nitrate, and replaced by hydrogen (H^+). Other drivers of acidification include acid precipitation (Rechcigl and Sparks, 1985, Vanbreemen et al., 1983) and nitrification. Microorganisms can also generate organic acids and nitrate from the decomposition of plant residues which also contribute the soil acidification. Conversely low pH and aluminium mobilization can affect the microbial populations (Fierer and Jackson, 2006) which are required for stubble turnover and nutrient recycling.

Approximately 30% of total land area consists of acid soils, and almost 70% of the world's potentially arable lands are acidic (Ma and Ryan, 2010, Vonuexkull and Mutert, 1995). The two main geographical belts of acid soils include the humid northern temperate zone mainly covered by coniferous forests and the humid tropics which support savanna and tropical rain-forests.

The American continent, Asia, Africa, Europe and Australia and New Zealand account for 40.9%, 26.4%, 16.7%, 9.9% and 6.1% of the world's acid soil respectively. Most acid soils in Asia are distributed throughout Southeast Asia and the Pacific. In Africa large tracts of the acid soil cannot be used for cultivation because they are sandy, nutrient-deprived and water-limited (Vonuexkull and Mutert, 1995).

Naturally acidic soils occupy about one third of Australia, but many agricultural soils in the intensive land-use regions have become more acidic as the result of removal of harvestable product, leaching of nitrate and calcium from nitrogen-producing pastures (Australia State of the Environment report, 2001), and high

applications of nitrogen fertilizer (Juo *et al.*, 1995, Matsuyama *et al.*, 2005, Sirovy, 1979). Rapid acidification associated with the overuse of nitrogen fertilizer is also an emerging problem in China (Guo *et al.*, 2010). Extremely acid soils can mobilise and increase the bioavailability of other toxic metals such as lead, mercury, zinc, copper, cadmium, chromium, manganese, and vanadium. All these factors may affect plant growth as well as the ecology of soil bacteria, mosses, algae, fungi, and invertebrates.

1.2 Aluminium toxicity

Acid soil are often low in basic cations and prone to crusting, erosion and compaction. Nevertheless these nutrient deficiencies and physical constraints are rarely the main reasons for poor growth of crop plants on these soils. Instead, soluble Al^{3+} in acid soil is the major factor limiting growth because it inhibits root growth at very low concentrations. Indeed the inhibition of root growth is the primary symptom of plant stress on acid soils (Munns, 1965). There are exceptions because many plants endemic to tropical and sub-tropical regions cope well and even thrive on acid soils. The growth of these species can even be stimulated by Al^{3+} and some accumulate high concentrations in their leaves. These are discussed in more detail later.

Al^{3+} can begin to inhibit root growth of wheat (*Triticum aestivum* L.) within minutes or hours in simple hydroponic solutions (Ryan *et al.*, 1992). Longer exposures result in thickened roots, damage root cap, and lesions in the epidermal and cortical tissues near the apices. The root system becomes small and damaged which limits water and nutrient acquisition. Root apices are the most sensitive part of root and Al^{3+} must contact the apices directly for growth to be affected (**Figure 1.1**). Exposure of an entire maize root to Al^{3+} except the apical 5 mm did not affect growth in the short term (Ryan *et al.*, 1993). Within this apical region the zone between the meristematic and elongation zones (distal transition zone) appears to be the most sensitive part of the root (Sivaguru and Horst, 1998). The concentration-dependent responses of root growth vary between species and even among genotypes. In some plants root growth remains unaffected at low concentrations of Al^{3+} but declines once a threshold is reached. This is called the *threshold for toxicity* response (Barcelo and Poschenrieder, 2002). In other species root growth is

stimulated by low concentrations of Al^{3+} but declines at higher concentrations. This *hormesis-type* response, is interpreted as Al^{3+} first alleviating H^+ toxicity at low concentrations and then becoming toxic itself at higher concentrations. A third response observed shows growth inhibition at low concentrations of Al^{3+} (or short exposures) but little or no effect at higher concentrations (or longer exposures). This is called the *threshold for tolerance* model and is indicative of an acclimation response.

For many crops including the cereals, most of the Al^{3+} absorbed by roots is located in the apoplast. The fixed negative charges on the membrane surfaces and pectin in the cell walls attract and bind cations, and especially highly-charged cations like Al^{3+}. Nevertheless it is still uncertain whether this apoplastic Al^{3+} is toxic to plants or if Al^{3+} needs to enter the cytosol to cause injury. By binding to pectin in the cell walls Al^{3+} can rigidify the walls and restrict solute flow through the apoplast (Horst *et al.*, 2010, Sivaguru *et al.*, 2006). High concentrations of Al^{3+} in the apoplast can induce callose production (1,3 beta D-glucan) and affect membrane function by binding with lipids and proteins or by displacing calcium from critical sites on membranes (Foy *et al.*, 1978). Al^{3+} can also directly inhibit nutrient uptake by blocking the function of ion channels involved in Ca^{2+} and K^+ influx (Gassmann and Schroeder, 1994, Pineros and Tester, 1993).

Cytosolic levels of free Ca^{2+} ($[Ca]_c$) are typically below 1.0 μM in most living cells but transient increases act as signals to control cellular functions and responses to hormones and stress. Ca^{2+}-sensitive fluorescent compounds have detected transient increases in $[Ca]_c$ in root cells treated with Al^{3+} (Rincon-Zachary *et al.*, 2010). The rapidity of these responses indicate that Al^{3+} is causing damage in the apoplast and that cytosolic Ca^{2+} could signal early responses to Al^{3+} stress. Al^{3+} can interfere with another signal transduction pathway involving inositol 1,4,5-trisphosphate (Jones and Kochian, 1995) as well as actin and tubulin stability (Grabski and Schindler, 1995, Sivaguru *et al.*, 2003b).

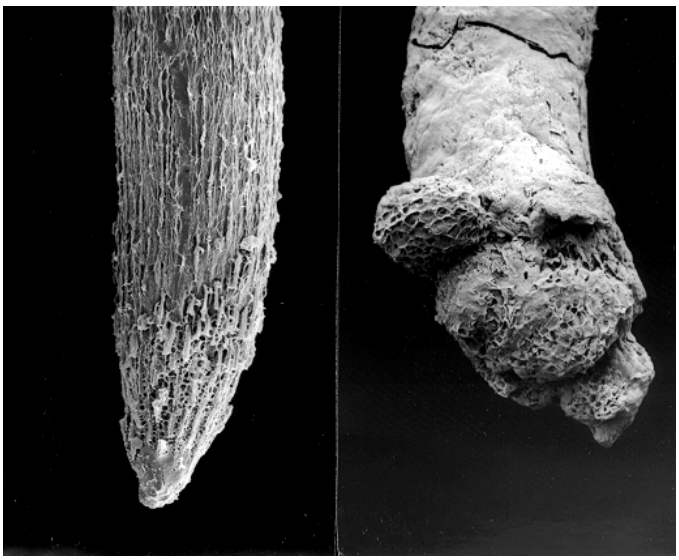


Figure 1.1 Effect of Al^{3+} toxicity on roots (Reprinted from Delhaize and Ryan 1995). The scanning electronmicrographs show root apices of near-isogenic wheat plants, ET8 and ES8, that differ in Al^{3+} resistance at a single major locus. The plants were grown for 4 days growth in 0.2 mM $CaCl_2$ (pH 4.3) with 5 μM $AlCl_3$. The resistant line (ET8, on the left) is unaffected by the treatment whereas the sensitive line (ES8, on the right) shows considerable damage to its tissues. The greater Al^{3+} resistance of ET8 is controlled by the *TaAMLT1* gene on chromosome 4DL. *TaALMT1* encodes an Al^{3+}-activated anion channel that facilitates malate efflux from the root apices (Sasaki *et al.*, 2004).

Small but measureable amounts of Al^{3+} does enter the cytosol perhaps via non-specific cation channels (Lazof *et al.*, 1994, Rengel and Reid, 1997, Taylor *et al.*, 2000). The combination of pH, ionic strength and availability of organic ligands in the cytosol maintain the soluble Al^{3+} concentrations to extremely low levels, perhaps less than 1 nM. However even these concentrations may cause damage because Al^{3+} can out-compete other cations like Mg^{2+} and Ca^{2+} for important binding sites and even bind with DNA (Martin, 1992). Al^{3+} also triggers oxidative stress in root cells by triggering the production of reactive oxygen species (Yamamoto *et al.*, 2001). Whether this response is induced by apoplastic Al^{3+} or symplastic Al^{3+} is unclear but these highly reactive compounds can rapidly damage membranes, proteins and nucleic acids. Oxidative stress induces callose production which in turn increases cell wall rigidity and decreases the symplastic flow of solutes via the plasmadesmata (Horst et al., 2010, Sivaguru *et al.*, 2000).

In summary, Al^{3+} interferes with many cellular functions. The Al^{3+}-induced changes to cytosolic Ca^{2+} concentration, oxidative stress and callose production are likely to signal the early signs of Al^{3+} injury.

1.3 Natural variations

Plant species vary widely in their ability to grow and yield on acid soils (Foy, 1988). Mclean and Gibert (1927) investigated the relative Al^{3+} resistance of several crop plants. Sensitive crops included *Lactuca sativa* (lettuce), *Beta vulgaris* (beet), *Phleum pratense* (timothy), *Hordeum vulgare* (barley), moderately resistant crops were *Raphanus sativus* (radish), *Sorghum bicolor* (sorghum), *Brassica oleracea* (cabbage), *Avena sativa* (oat), *Secale cereale* (rye) and noticeably resistant species included *Zea mays* (maize), *Brassica rapa* (turnip) and *Agrostis gigantean* (redtop) (McLean and Gilbert, 1927). Among the cereals rice is significantly more resistant than maize, wheat and sorghum, while barley and durum wheat are among the most sensitive cereal species (Famoso *et al.*, 2010, Garvin and Carver, 2003, Khatiwada *et al.*, 1996).

Significant variation in Al^{3+} resistance occurs within many species as well including maize, wheat, barley, rice, sorghum, snapbean and *Arabidopsis* (Foy, 1988, Foy *et al.*, 1993, Furlani *et al.*, 1987, Kochian et al., 2004, Koyama *et al.*, 2003, Magalhaes

et al., 2007, Ryan *et al.*, 2011, Toda *et al.*, 1999). This variation provides opportunities for breeders to develop new cultivars better suited to acid soils. Even barley, which is considered one of the most Al^{3+}-sensitive members of the small-grained *Triticeae*, displays significant genotypic variability. A seedling-based screen of barley lines in the South and East Asian Barley Core Collection identified Kearney and Golden Promise as sensitive to Al^{3+} while Dayton and several Japanese cultivars (Honen, Ohichi and Zairai Tanbo) were among the most resistant (Moroni *et al.*, 2010).

A greater variation occurs in hexaploid or bread wheat where differences in root growth can vary by ten-fold or more in short-term growth assays or in field screens (Bona *et al.*, 1993, Cosic *et al.*, 1994, Dai *et al.*, 2009, Foy, 1996, Garvin and Carver, 2003, Pinto-Carnide and Guedes-Pinto, 1999, Raman *et al.*, 2008, Rengel and Jurkic, 1992, Ryan *et al.*, 1995a, Tang *et al.*, 2003). Highly Al^{3+}-resistant genotypes of bread wheat commonly used in experiments include BH1146 and Carazinho from Brazil and Atlas 66 from the USA. In most cases enhanced Al^{3+} resistance is associated with reduced Al^{3+} accumulation in the roots. Therefore the more resistant genotypes of wheat, maize, barley, sorghum and rye are able to exclude Al^{3+} from their roots cells – especially from the root apices.

1.4 Genetics of Al^{3+} resistance

The inheritance and genetics of Al^{3+} resistance have been widely studied in members of the *Triticeae*. In wheat, barley and sorghum Al^{3+} resistance is often consistent with a single genetic locus inheritance while in maize and rice it is a more complex multigenic trait.

1.4.1 Single or few genes: cases of simple inheritance

Crop improvement programs in Brazil and the US led to the development of highly-resistant cultivars of wheat such as BH1146 and Atlas66 which were used for genetic mapping and quantitative trait loci (QTL) analyses. Many studies indicate a single locus controls most of the variation in Al^{3+} resistance. For instance, a population of recombinant inbred lines developed with BH 1146 and the sensitive cultivar Anahuac showed a bimodal distribution for Al^{3+} resistance, consistent with single gene inheritance. Similar results were obtained with other populations

(Raman *et al.*, 2005). The resistance locus in BH 1146, named Alt_{BH}, was mapped to chromosome 4DL and explained 85% of the phenotypic variation (Riede and Anderson, 1996). The location of Alt_{BH} gene was further confirmed using 91 recombinant inbred lines and a set of wheat deletion lines (Milla and Gustafson, 2001) and Luo et al. (1996) had also linked this chromosome to Al^{3+} resistance using Chinese Spring deletion lines. More recently a major Al^{3+} resistance gene called *TaALMT1* (**Figure 1.1**; see later) was mapped to the same locus on 4DL (Raman et al., 2005) while minor loci were mapped to 4BL and 3BL (Navakode *et al.*, 2009, Ryan *et al.*, 2009).

QTL analyses of 100 F_2 barley seedlings derived from the Al^{3+}-resistant cultivar (Murasakimochi) and the Al^{3+}-sensitive cultivar (Morex) identified a single Al^{3+} resistance gene on chromosome 4H which explained more than 50% of the phenotypic variation (Ma *et al.*, 2004). The *Alp* locus was also mapped to chromosome 4H in a high-resolution map generated from a double haploid population derived from Dayton and Zhepi 2 (Raman *et al.*, 2003, Tang *et al.*, 2000, Wang *et al.*, 2007).

Sorghum is closely related to maize and possesses the second smallest genome among cultivated grasses (Mullet *et al.*, 2002). Like wheat and barley the genetics indicate that a single locus, Alt_{SB}, on chromosome 3 controls most of the variation in resistance (Magalhaes *et al.*, 2004).

1.4.2 Multiple genes: cases of complex inheritance

Rye is generally regarded as a highly resistant species. Unlike wheat, rye is self-incompatible, so co-segregation experiments in rye generally detect a number of Al^{3+} resistance loci. Using wheat-rye addition lines, Aniol and Gustafson (1984) identified at least three different Al^{3+} resistance loci, *Alt1* on chromosome 6RS, *Alt2* on 3RS, and *Alt3* on 4RL. A new locus named *Alt4* was located on chromosome 7RS (Matos *et al.*, 2005). More recently the 7RS locus was shown to include a cluster of *ALMT*-like genes in the resistant lines (see later) (Collins *et al.*, 2008).

More than 30 QTLs for Al^{3+} resistance have been reported in rice using populations derived from *indica* and *japonica* cultivars as well as wild relatives like *Oryza rufiogon* (Ma *et al.*, 2002, Nguyen *et al.*, 2003, Nguyen *et al.*, 2001, Nguyen *et al.*,

2002, Wu *et al.*, 2000, Xue *et al.*, 2007, Xue *et al.*, 2006a, Xue *et al.*, 2006b). Resistance loci on chromosomes 1, 8 and 9 were consistently identified in these studies which confirm resistance is a multi-genetic trait in this species. Given the conservation of genetic locations among the *Triticeae* (synteny), it will be intriguing whether orthologous loci to the resistance loci from other cereals play a similar role in rice. For instance, a major resistance locus on rice chromosome 3 is homeologous to *Triticeae* 4L where the Al^{3+} resistance loci on wheat and barley are located **(Nguyen et al., 2003)**. In maize, five QTLs on chromosomes 2, 6 and 8 contribute to Al^{3+} resistance and these explain 60% of the phenotypic variation. Dominant and additive effects were detected between these loci (Ninamango-Cardenas *et al.*, 2003).

1.5 Mechanisms of Al^{3+} resistance

Some plants have evolved mechanisms that enable them to tolerate Al^{3+} toxicity and acid soils better than others. The identification and characterization of these mechanisms has been the focus of considerable research. Some very resistant species like tea (*Camelia sinensis*) and *Hydragenea* sp accumulate high concentrations of Al^{3+} in their roots and leaves while others, such as resistant members of the *Triticeae*, exclude Al^{3+} from their root and shoots. For instance the concentration of Al^{3+} in the root apices of an Al^{3+}-sensitive wheat cultivar after 24 h in 50 μM Al^{3+} was 10-fold greater than a resistant cultivar (Rincon and Gonzales, 1992) and similar results were reported in closely-related wheat lines that differed in Al^{3+} resistance (Delhaize *et al.*, 1993a). Therefore two main mechanisms have been proposed to account for this resistance: exclusion mechanisms and tolerance mechanisms, and evidence is now available for both of these. Exclusion mechanisms prevent Al^{3+} from entering the cytosol and minimize harmful interactions from occurring in the apoplast. Tolerance mechanisms allow plants to safely take-up and accumulate Al^{3+} within their cells. Both mechanisms may be operating in the same plant.

1.5.1 Mechanisms of Al^{3+} exclusion

There are several ways Al^{3+} could be prevented from accumulating in apoplastic and symplastic fractions of root tissues. Cell wall chemistry could affect Al^{3+} binding, the maintenance of a slightly higher rhizosphere pH could shift the

hydrolysis of soluble aluminium from Al^{3+} to $Al(OH)^{2+}$ which would reduce accumulation in the cell wall, compounds could be released from the root which bind the harmful Al^{3+} and limit other more damaging interactions from occurring and Al^{3+} could be actively exuded from the root cell by some active transport process. Charged residues on cell wall pectin will attract and accumulate cations but pectin content is not consistently correlated with either Al^{3+} sensitivity or resistance (Horst et al. 2010). Recent studies showing that methylation of the pectin residues is correlated with reduced Al^{3+} accumulation in the wall support the idea that modifications to cell walls can increase Al^{3+} resistance.

Currently there are no examples of resistance based on Al^{3+} exudation and nor are there convincing cases linking higher rhizospheric pH to genotypic variation in resistance despite detailed studies in wheat and maize (Pineros *et al.*, 2005). However there are claims of an Al^{3+}-resistant *Arabidopsis* mutant (*alr-104*) showing a pH dependent increase in resistance (Degenhardt *et al.*, 1998). Measurements with micro-pH electrodes detected a 0.15 unit higher pH at the root surface of *alr-104* plants compared to wildtype plants and subsequent experiments indicated this relatively small pH change could explain the increased resistance. The molecular biology of the *alr-104* mutation has not been characterized in detail.

The importance of Al^{3+} exclusion to the very high resistance of rice was confirmed after characterizing two Al^{3+}-sensitive mutations, *als1* and *c68,* because both of these recessive mutations lead to increased accumulation of Al^{3+} in the roots (Huang *et al.*, 2009, Ma *et al.*, 2005). *als1* carries a mutation in a gene encoding part of an ATP binding cassette (ABC) transporter (see later) while the *c68* mutation remains uncharacterized at the genetic level.

The exclusion mechanism for which most supporting evidence is available is the release of organic anions from roots (Delhaize *et al.*, 2007, Ma, 2000, Ma *et al.*, 2001, Ryan *et al.*, 2001). Malate and citrate are the two anions most commonly reported but oxalate efflux occurs from a few species. Once these anions are released from root cells they bind the Al^{3+} and prev ent it from accumulating in the apoplast, damaging the cells and being absorbed by the roots. Efflux is largely restricted to the root apices and in nearly all cases it does not occur continuously but is activated by exposure to Al^{3+}. The effectiveness of these anions in reducing Al^{3+}

toxicity is demonstrated by adding them to solutions containing toxic concentrations of Al^{3+}. Root growth improves as the anion concentration increases. This occurs for malate, citrate and oxalate additions but not for anions, such as succinate and acetate, which have lower stability constants for Al^{3+} (Ryan et al., 2001). This exclusion mechanism has now been reported in species from the Poaceae (e.g. wheat, barley, sorghum, maize and rye), Araceae (e.g. taro), Polygonaceae (e.g. buckwheat), Brassicaceae (e.g. *Arabidopsis*) and the Fabaceae (e.g. soybean, snapbean, common bean, *Cassia tora*).

The first study linking organic anion efflux with Al^{3+} resistance was described by Miyasaka et al. (1991). They showed that Al^{3+} activated citrate exudation from snapbean roots and that the efflux from a resistant cultivar was 10-fold greater than efflux from a sensitive cultivar. Another example was reported soon after in wheat by Delhaize et al.(1993b) and Ryan et al. (1995a) using near-isogenic wheat lines differing in Al^{3+} resistance. These studies showed that addition of Al^{3+} to a nutrient solution rapidly stimulated malate release from the root apices of the resistant iso-line but not from the sensitive line. This rapid activation of efflux is termed a Type I response (**Figure 1.2**). Type I responses are interpreted as Al^{3+} activating a transport protein already present in the plasma membrane so little or no delay occurs (Ma et al., 2001). An F_2 population generated from these near-isogenic lines demonstrated that resistance co-segregates with malate efflux. Subsequent analyses revealed a strong positive correlation between malate efflux and Al^{3+} resistance in diverse germplasm which supports the importance of this major trait in wheat (Raman et al., 2005, Ryan et al., 1995a, b). Organic anion efflux does not appear to be important contributor to the high resistance of rice but it does appear to be a minor contributor in maize. Several maize genotypes display an Al^{3+}-activated efflux of citrate but the efflux is delayed by several hours after Al^{3+} addition. This is referred to as a Type II response (**Figure 1.2**). The delay is interpreted as Al^{3+} first inducing expression of the transport protein before then activating anion efflux (Ma et al., 2001). Type II responses have also been reported for citrate efflux from *Cassia tora* and rye (Ma et al., 2001). Some maize genotypes also show a slower Al^{3+}-inducible increase of citrate content suggesting that Al^{3+} resistance may also rely on internal detoxification (Pineros *et al.*, 2002). Nevertheless no clear correlation has been established between citrate exudation and Al^{3+} resistance

among a large range of maize genotypes (Pineros et al., 2002) which supports a model where several different mechanisms contribute to Al^{3+} resistance in this species.

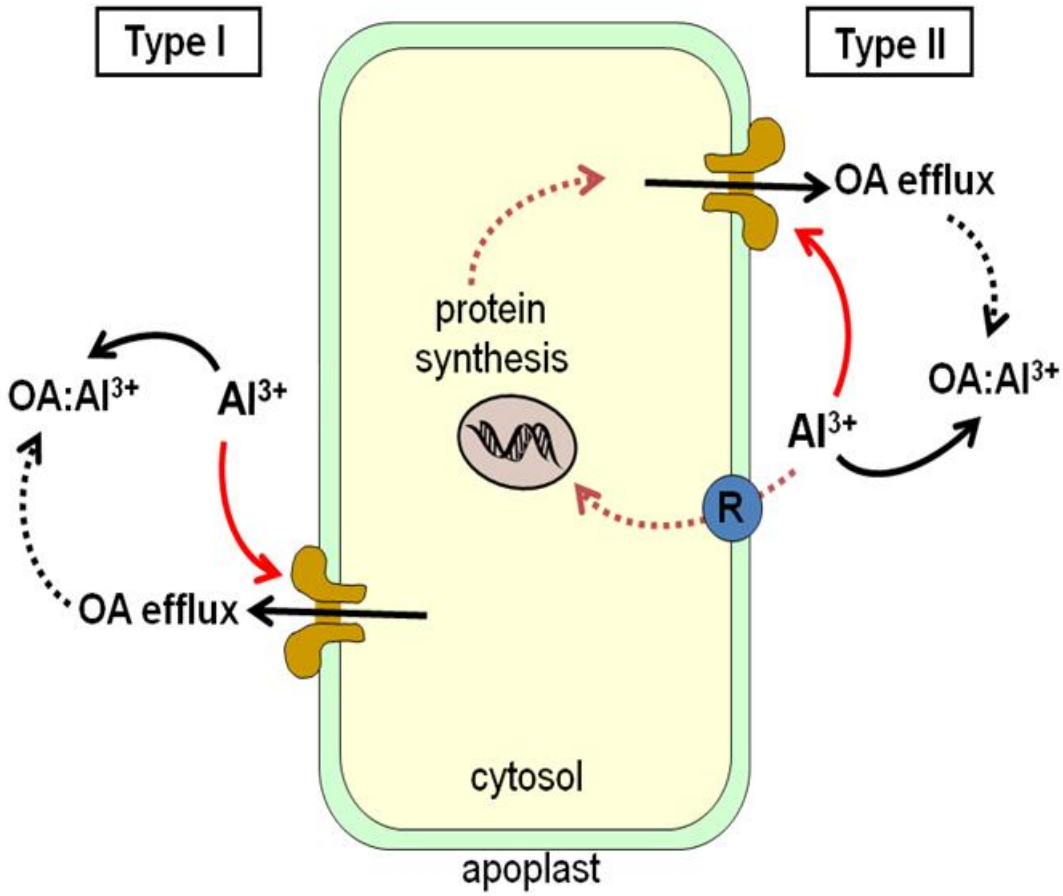


Figure 1.2 Al^{3+}-activated organic anion efflux. The Type I response illustrates the rapid activation of organic anion efflux in species such as wheat where the anion channel is constitutively expressed. Al^{3+} is able to rapidly activate efflux by interacting directly with the pre-existing proteins (red arrows). The Type II response occurs in maize and rye and shows a delay between the addition of Al^{3+} and the start of organic anion efflux. This delay is interpreted as Al^{3+} first inducing the expression of the transport protein via a signal transduction pathway possibly involving a specific receptor ("R")(blue arrows). Once synthesized and inserted in the plasma membrane, Al^{3+} is thought to interact with the protein to activate efflux of organic anion (OA).

1.5.2 Mechanisms of Al^{3+} tolerance

Instead of excluding Al^{3+} from their tissues, many highly-tolerant species absorb Al^{3+} and store it in their leaves sometimes to concentrations exceeding 3000 mg/kg. This relies on quite different processes involving complexation, detoxification and transport of aluminium within the plant. Aluminium accumulator species are defined as those with 1000 mg/kg aluminium or more in their leaves. Some of these species include tea (*Camelia sinensis*), *Hydrangea sp* and buckwheat (*Fagopyrum esculentum*) as well as a range of tree and shrub species (Haridasan and Dearaujo, 1988). Most of the aluminium in tea leaves resides in the apoplast (Tolra *et al.*, 2011) whereas in the leaves of *Hydrangea* and buckwheat the aluminium is bound in vacuoles by citrate and oxalate anions, respectively. *Hydrangea* is an ornamental plant that changes the colour of its flowers from pink to blue when grown in acid soils with high Al^{3+} availability (Ma *et al.*, 1997a). This colour change is caused by the formation of aluminium delphinidin complexes or aluminium caffeolylquinate complexes (Takeda *et al.*, 1985). Buckwheat can accumulate 15,000 mg/kg aluminium in its leaves when grown in acid soils (Ma et al., 1997a).

High shoot accumulation of aluminium implies soluble aluminium is transported through the xylem and then stored safely in leaf vacuoles or in the apoplast. To protect the plant cells from damage aluminium is bound by organic ligands as it is transported throughout the plant. ^{27}Al NMR studies identified aluminium oxalate complexes (1:3) in buckwheat leaves (Ma et al., 2001), but aluminium citrate complexes in the xylem (Ma and Hiradate, 2000). It appears that aluminium undergoes a ligand exchange with oxalate and citrate depending on whether it is transported into xylem or being sequestered in the leaves.

1.6 Identification of Al^{3+} resistance genes in plants

Several Al^{3+} resistance genes have now been mapped and cloned from a range of species (**Table 1.1**). Ryan et al. (2011) classifieds these resistance genes into three groups: (1) those isolated by analysing segregating populations and therefore explain genotypic variation, (2) those identified from mutant analysis and therefore do not necessarily explain genotypic variation, and (3) likely resistance genes which require additional supporting information.

Table 1.1 Al^{3+} resistance genes in plants

Species	Genes	Protein Function	Reference
Organic transporters			
Wheat	*TaALMT1*	Malate transporter	(Sasaki et al., 2004)
Arabidopsis	*AtALMT1*	Malate transporter	(Hoekenga *et al.*, 2006)
Sorghum	*SbMATE*	Citrate transporter	(Magalhaes et al., 2007)
Barley	*HvAACT1*	Citrate transporter	(Furukawa *et al.*, 2007)
Rye	*ScALMT1 gene cluster*	Malate transporter	(Collins et al., 2008)
Arabidopsis	*AtMATE1*	Citrate transporter	(Liu *et al.*, 2009)
Maize	*ZmMATE1*	Citrate transporter	(Maron *et al.*, 2010)
ABC transporters and other proteins			
Arabidopsis	*AtSTOP1*	C_2H_2-type Zn finger transcription factor	(Iuchi *et al.*, 2007)
Arabidopsis	*AtSTAR1*	ABC transporter-basic detoxification of Al	(Huang *et al.*, 2010)
Arabidopsis	*ALS1*	Half ABC transporter	(Larsen *et al.*, 2007)
Arabidopsis	*ALS3*	Half ABC transporter	(Larsen *et al.*, 2005)
Rice	*ART1*	C_2H_2-type Zn finger transcription factor	(Yamaji *et al.*, 2009)
Rice	*STAR1,STAR2*	ABC transporter-UDP-glucose transport	(Huang et al., 2009)
Likely Al^{3+} resistance gene			
Wheat	*TaMATE1*	Citrate transporter	(Ryan et al., 2009)
Rye	*ScMATE2*	Citrate transporter	(Yokosho *et al.*, 2010)
Brassica napus	*BnALMT1* *BnALMT2*	Malate transporter Malate transporter	(Ligaba *et al.*, 2006)

1.6.1 Organic anion transporters

The genes controlling organic anion efflux from roots were the first Al^{3+} resistance genes to be isolated from plants. Those controlling malate efflux belong to the *ALMT* (aluminium activated malate transporter) family of genes and those controlling citrate efflux belong to the *MATE* (multi-drug and toxic compound extrusion) family of genes. The genes controlling oxalate efflux are still unknown. The first Al^{3+} resistance gene cloned from plants was the wheat gene *TaALMT1* which encodes an Al^{3+}-activated malate channel (Sasaki et al., 2004). *TaALMT1* was identified by cDNA subtractive hybridization using near-isogenic wheat lines ET8 (resistant) and ES8 (sensitive). *TaALMT1* was identified for being more highly expressed in root tips of ET8 than of ES8 and its expression co-segregated with Al^{3+} resistance in a segregating population. Heterologus expression of *TaALMT1* in *Xenopus laevis* oocytes, tobacco suspension cells, barley, wheat and *Arabidopsis* all generate the same phenotype: an Al^{3+}-activated efflux of malate (Sasaki et al., 2004). Al^{3+} resistance was not related to the coding alleles of *TaALMT1*, but to the level of expression (Raman et al., 2005). Polymorphisms detected in the promoter of *TaALMT1* are correlated with Al^{3+} resistance. Tandem repeats in the promoter of resistance genotypes explain the higher expression in resistant plants such that the larger the number of repeats the higher the expression and this is correlated with greater malate efflux (Ryan *et al.*, 2010, Sasaki *et al.*, 2006). Homologues of *TaALMT1* also control Al^{3+} resistance in *Arabidopsis* (Hoekenga et al., 2006) and rye (Collins et al., 2008). However not all *ALMT* genes confer Al^{3+} resistance because members of this family in barley and *Arabidopsis* (*HvALMT*, *AtALMT9* and *AtALMT12*) have other functions on the tonoplast of leaves and in guard cells (Gruber *et al.*, 2011, Kovermann *et al.*, 2007, Sasaki *et al.*, 2010).

The first *MATE* gene involved in Al^{3+} resistance was cloned in sorghum by positional cloning (Magalhaes et al., 2007). *SbMATE* encodes a transport protein located on the plasma membrane that facilitates citrate release from the root cells. *SbMATE* is constitutively expressed in the root apices of resistant sorghum lines but Al^{3+} treatment increases expression over hours and days and this change parallels the increase in citrate efflux. Interestingly, the coding regions of *SbMATE* in the sensitive and resistant genotypes are identical, with polymorphisms in one of the

introns only. It will be interesting to discover how *SbMATE* expression is controlled in sorghum.

MATE genes also control the citrate efflux from Al^{3+}-resistant barley plants and *Arabidopsis*. The *HvAACT1* gene from barley (also known as *HvMATE*) was also isolated by positional cloning (Furukawa et al., 2007). Like *TaALMT1* in wheat, *HvAACT1* is constitutively expressed in roots but Al^{3+} is still required to activate citrate efflux. Unlike *TaALMT1* and *SbMATE*, *HvAACT1* expression is higher slightly behind the root apices which may influence its effectiveness. Liu et al. (2009) showed that knock-out mutations in *AtMATE* prevent the Al^{3+}-activated efflux of citrate but the contribution of *AtMATE* to the resistance of this species is relatively small compared to the malate channel AtALMT1.

Al^{3+} resistance in maize is likely to involve several mechanisms. Nevertheless citrate efflux does contribute and Maron et al. (2010) isolated a *MATE* gene called *ZmMATE1* which co-localizes with a major QTL for Al^{3+} resistance. *ZmMATE1* is mainly expressed in roots, is up-regulated by Al^{3+} and shows higher expression in Al^{3+}-resistant genotypes. *ZmMATE1* elicits anion efflux when expressed in *Xenopus oocytes* and measurements with labeled [^{14}C]-citrate confirmed ZmMATE1 transports citrate (Maron *et al.*, 2010).

Other candidate genes are likely to control Al^{3+} resistance but need confirmation. For instance, *TaMATE1* expression in wheat segregates with citrate efflux in some Brazillian genotypes but it needs to be demonstrated directly that TaMATE is a citrate transporter (Ryan et al., 2009). The citrate efflux from these few genotypes of wheat differs from other species in one important way: it occurs constitutively and does not require Al^{3+} to activate it. Two genes from *Brassica napus*, *BnALMT1* and *BnALMT2*, encode functional malate transporters in *Xenopus* oocytes and their expression is induced by Al^{3+} but no genetic analysis or knockout mutants have confirmed that they contribute to Al^{3+} resistance.

1.6.2 Other resistance genes

A different set of Al^{3+} resistance genes was identified using mutant analysis (**Table 1.1**). This approach requires no prior knowledge regarding genetics or mechanisms involved. Mutagenized seed is generated by chemical treatments, radiation or the

random insertion of a DNA fragment (T-DNA or transposon) into the genome. M2 seedlings are screened and those that grow similar to wild-type plants under control conditions, but show altered responses to Al^{3+} stress, are selected for further Al^{3+} resistance analysis. Candidate genes can be isolated by mapping or obtaining the sequence flanking the T-DNA region and analyzed further. The candidate genes can be characterized by overexpression studies, knockout studies, mutant analysis or association analysis. These genes need not show allelic variation within natural populations.

Using this approach Huang et al. (2009) cloned two genes from rice called *STAR1* and *STAR2* (sensitive to Al^{3+} rhizotoxicity) which cause plants to be hypersensitive to Al^{3+} toxicity when knocked out. Both genes are expressed in roots and induced by Al^{3+} treatment. *STAR1* encodes a nucleotide binding domain of bacterial-type ATP binding cassette (ABC) transporter and *STAR2* encodes the transmembrane domain for an ABC transporter. Huang et al (2009) demonstrated that *STAR1* and *STAR2* interact to form a functional ABC transporter which localizes to vesicles of most root cells except for those in the epidermal layer of the mature zone. *Xenopus laevis* oocytes expressing *STAR1/STAR2* can transport UDP-glucose but a more recent study shows that STAR1 is also involved in nicotianamine transport, a secondary metabolite used for the long-distance transport of Fe^{3+} in plants. The role of STAR1/STAR2 in Al^{3+} resistance remains unclear but it could be involved with releasing compounds that modify the cell wall during Al^{3+} stress.

A homologue of *STAR1* in *Arabidopsis*, called *AtSTAR1*, also encodes an ATP-binding domain of a bacterial-type ABC transporter (Huang et al., 2009). A line carrying a knockout mutation showed increased sensitivity to Al^{3+} and early flowering. Unlike *OsSTAR1*, *AtSTAR1* is expressed in both the roots and shoots, and its expression is not induced by Al^{3+} stress. *AtSTAR1* may interact with another protein called ALS3 to form a functional ABC transporter. *ALS3* had been identified previously in similar screens in *Arabidopsis* because plants carrying loss-of-function mutations are more sensitive to Al^{3+} stress (Larsen et al., 2007, Larsen et al., 2005).

STOP1 (sensitive to protons) encodes a transcription factor identified by analysing *Arabidopisis* mutants which are hypersensitive to H^+ toxicity. STOP1 belongs to

C_2H_2-type zinc finger family of proteins. *stop1* mutants are more sensitive to Al^{3+} but not to a range of other cations including cadmium, copper, lanthanum, manganese and sodium. STOP1 is required for the induction of a range of genes including *AtALMT1* which encodes the malate transporter. *STOP1* plays a critical role in enabling *Arabidopsis* to resist stress induced by low pH and Al^{3+} toxicity (Iuchi et al., 2007).

1.7 Transgenic approaches for increasing Al^{3+} resistance

The increasing demands for food from a growing world population highlight the need to overcome the major soil constraints currently limiting crop yields. For acid soils, the application of lime (calcium carbonate) can increase the soil pH but this usually only changes the surface pH in the year of application and it can take decades for acidity to be neutralized at depth. Additionally, in third world countries it can be prohibitively expensive to apply sufficient lime to neutralize soil acidity. Increasing the acid soil tolerance by conventional breeding has been successfully applied to several crop species and this complements liming practices as a way of managing acid soils. However, some species lack sufficient variation in their germplasm and genetic modification provides another avenue for increasing their acid soil tolerance. As described above the mechanisms of Al^{3+} resistance in species, such as wheat, sorghum and barley have been elucidated and the genes underlying these mechanisms have been isolated. These genes have been used to generate transgenic plants with enhanced Al^{3+} resistance. A range of other genes, not necessarily responsible for natural variation in Al^{3+} tolerance, have also been used to enhance the Al^{3+} resistance of plants. The following discussion summarizes these recent attempts to enhance Al^{3+} resistance using biotechnology (**Table 1.2**).

1.7.1 Over-expression of genes involved in organic anion biosynthesis

The important role of organic anion efflux in Al^{3+} resistance was established 20 years ago and more than a decade before the genes controlling this trait were cloned. Therefore the first attempts to increase organic anion efflux to improve Al^{3+} resistance focused on increasing organic anion synthesis because the key enzymes and genes involved in those pathways were well known (**Table 1.2**). This approach was based on the idea that an increased concentration of organic anions in the

cytosol would result in increased organic anion transport across the plasma membrane. The underlying assumption was that transport of organic anions across the plasma membrane is not the rate-limiting step for efflux. Citrate synthase was a sensible starting point due to the known role of citrate in the Al^{3+} resistance of *Cassia tora*, maize, rye and snapbean (Ryan et al., 2001; Ma et al., 2001). Citrate synthase is the first enzyme involved in the tricarboxylic acid and glyoxylate cycles. De la Fuente et al. (1997) transformed tobacco and papaya with the citrate synthase gene (CS) from the bacterium *Pseudomonas aeruginosa* to increase the biosynthesis and efflux of citrate for enhanced Al^{3+} resistance. When homozygous lines of tobacco expressing the CS gene were analyzed they were found to accumulate up to 10 fold more citrate than the wildtype plants. Citrate efflux of the transgenics was increased four-fold over wildtype and this was associated with enhanced Al^{3+} resistance. Similar results were reported for transgenic papaya expressing the same transgene. However, subsequent work by Delhaize et al. (2001) was not able to repeat these findings on the same tobacco lines or even when the gene was expressed to a much greater level. More recently other groups have reported enhanced Al^{3+} resistance when CS expression was increased in alfalfa (Barone *et al.*, 2008), *Arabidopsis* (Koyama *et al.*, 2000, Koyama *et al.*, 1999) and tobacco (Han *et al.*, 2009). In most of these cases the increases in Al^{3+} resistance were marginal except for tobacco transformed with a rice CS gene where transgenic lines showed up to 4.5-fold greater Al^{3+} resistance than the wildtype.

Malate dehydrogenase (MDH) which oxidises oxaloacetate to form malate is another enzyme involved in organic anion biosynthesis and this gene has now been over-expressed in several species. An MDH gene highly expressed in root nodules of alfalfa (*neMDH*) was over-expressed in alfalfa and this was associated with enhanced malate efflux and greater Al^{3+} resistance (Tesfaye *et al.*, 2001). Similarly, when MDH genes from *Arabidopsis* and *Escherichia coli* were expressed in tobacco, the transgenic plants showed enhanced malate efflux and improved Al^{3+} resistance (Wang *et al.*, 2010).

Table 1.2 Studies which have used biotechnology to increase Al^{3+} resistance in plants.

Gene function	Source of gene	Species transformed	Phenotype (RRG)	Reference
Organic anion metabolism				
Citrate synthase	*Pseudomonas aeruginosa*	Tobacco and papaya	2-fold	(de la Fuente et al., 1997)
Citrate synthase (*AtCS*)	*Arabidopsis*	Carrot	1.3-fold	(Koyama et al., 1999)
Citrate synthase (*DcCS*)	Carrot	*Arabidopsis*	1.2-fold	(Koyama et al., 2000)
Citrate synthase (*OsCS1*)	Rice	Tobacco	4.5-fold	(Han et al., 2009)
Citrate synthase	*Pseudomonas aeruginosa*	Alfalfa	2.5-fold	(Barone et al., 2008)
Citrate synthase (*AtmtCS*)	*Arabidopsis*	Canola	2-fold	(Anoop *et al.*, 2003)
Malate dehydrogenase	Alfalfa	Alfalfa	2-fold	(Tesfaye et al., 2001)
Malate dehydrogenase	Arabidospis *E coli.*	Tobacco	2.4-fold	(Wang et al., 2010)
Blue-copper-binding protein gene (*AtBCB*)	*Arabidopsis*	*Arabidopsis*	1.7-fold	(Ezaki *et al.*, 2000)
Stress response				
Glutathione S-transferase gene (*parB*)	Tobacco	*Arabidopsis*	1.7-fold	(Ezaki et al., 2000)
Peroxidase gene (*NtPox*)	Tobacco	*Arabidopsis*	1.7-fold	(Ezaki et al., 2000)
GDP-dissociation inhibitor gene (*NtGDI1*)	Tobacco	*Arabidopsis*	1.7-fold	(Ezaki et al., 2000)
Dehydroascorbate reductase	*Arabidopsis*	tobacco	1.5-fold	(Yin *et al.*, 2010)
Manganese superoxide dismutase	wheat	*Brassica napus*	2.5-fold	(Basu *et al.*, 2001)
Organic anion transporter				
TaALMT1	wheat	wheat	8-fold	(Pereira *et al.*, 2010)
TaALMT1	wheat	barley	20-fold	(Delhaize *et al.*, 2004)
TaALMT1	wheat	*Arabidopsis*	4-fold	(Ryan et al., 2011)
SbMATE	sorghum	*Arabidopsis*	2.5-fold	(Magalhaes et al. 2007)
Frd3	*Arabidopsis*	*Arabidopsis*	2-fold	(Durrett et al. 2007)
ZmMATE1	maize	*Arabidopsis*	3-fold	(Maron et al. 2010)
HvAACT1	barley	tobacco	2-fold	(Furukawa et al., 2007)

Phenotype (RRG) shows the reported increase in Al^{3+} resistance of the transgenic plants based on measurements of relative root growth (RRG).

1.7.2 Over-expression of genes involved in organic anion transport

Once the Al^{3+} resistance genes controlling organic anion efflux were identified and cloned they were transformed into plants (**Table 1.2**). These genes belong to the *MATE* or *ALMT* gene families and they encode transport proteins that mediate organic anion movement across the plasma membrane to the external medium.

TaALMT1 was the first gene encoding an organic anion transport protein involved in Al^{3+} resistance to be cloned from plants. *TaALMT1* has now been expressed in several species and in nearly all cases the transgenic plants and cells showed Al^{3+}-activated malate efflux and greater Al^{3+} resistance. The one exception was rice, where *TaALMT1* expression conferred Al^{3+}-activated malate efflux but not enhanced Al^{3+} resistance (Sasaki et al., 2004). The inability of *TaALMT1* to increase resistance in this case was attributed to the very high endogenous level of Al^{3+} resistance of this species.

Barley is among the most Al^{3+}-sensitive cereal crops but the small genotypic variation in resistance that does occur is correlated with low rates of citrate release, but not malate efflux (see above). Expression of *TaALMT1* in barley was associated with increased Al^{3+}-activated malate efflux and a significant increase in Al^{3+} resistance when compared to wildtype plants and null segregant lines (Delhaize et al., 2004). The transgenic barley showed enhanced Al^{3+} resistance when grown in both hydroponic culture and in acid soil. In hydroponic culture root growth of transgenics was more than 10-fold greater than wildtype (Delhaize et al., 2004). More recently it was shown that these transgenic barley had enhanced phosphorus-use efficiency and improved grain yield when grown on an acid soil (Delhaize *et al.*, 2009).

Similarly Al^{3+}-activated malate efflux and Al^{3+} resistance were enhanced when *TaALMT1* was over-experessed in wheat (Pereira et al., 2010) and *Arabidopsis* (Ryan et al., 2011). Some of the transgenic wheat lines displayed greater Al^{3+} resistance than ET8 (the source of the *TaALMT1* gene) in both hydroponic and soil experiments (Pereira et al., 2010).

MATE genes encoding citrate transporter proteins in sorghum (*SbMATE*), barley (*HvAACT1*), maize (*ZmMATE1*) and *Arabidopsis* (*AtMATE* and *Frd3*) were transformed into *Arabidopsis* or tobacco plants (Durrett et al. 2007; Furukawa et al. 2007; Magalhaes et al. 2007; Maron et al. 2010). *Frd3* is not an Al^{3+}-resistance gene but it does encode a transporter which releases citrate into the xylem to assist iron movement to the shoots. In all cases these genes increased citrate efflux and enhanced Al^{3+} resistance of the transgenic plants.

These findings indicate that the *MATE* and *ALMT* genes can be effectively used to enhance the Al^{3+} resistance of not only model species, but also important crop species. The observation that organic anion efflux can be increased by expression of a transport protein suggests that biosynthesis of organic anions is not a limiting factor for many plant species. To date, the transport proteins, and TaALMT1 in particular, have provided the most effective means to increase the Al^{3+} resistance of plants.

1.7.3 Genes not associated with organic anions

One of the first biotechnological strategies to increase Al^{3+} resistance sought to over-express genes induced by Al^{3+} stress, and especially those involved in combating oxidative stress.Ezaki et al. (2000) first identified a range of genes whose expression is induced by Al^{3+} and then overexpressed these genes in *Arabidopsis*. They found that an *Arabidopsis* blue-copper-binding protein gene (*AtBCB*), a tobacco glutathione S-transferase gene (*parB*), a tobacco peroxidase gene (*NtPox*) and a tobacco GDP-dissociation inhibitor gene (*NtGDI1*) conferred a degree of tolerance to $Al^{3+}$when over-expressed. In particular, overexpression of the *parB* gene simultaneously conferred resistance to both Al^{3+} and oxidative stresses. Other stress-related genes, such as dehydroascorbate reductase from *Arabidopsis* and manganese superoxide dismutase from wheat, were expressed in tobacco and *Brassica napus*, respectively with the transgenic plants showing enhanced Al^{3+} resistance (Basu et al., 2001, Yin et al., 2010). Overexpression of these stress-related genes in transgenic plants exhibited a 1.5-2.5-fold increase in relative root growth compared to wildtype.

Genes encoding proteins involved in various stress responses, endocytosis, lipid biosynthesis or Al-induced programmed cell death have also conferred a degree of Al^{3+} resistance when over-expressed in *Arabidopsis* or tobacco. These genes encode WAK1, a auxilin-like protein, a Δ8 sphingolipid desaturase and a Ced-2 protein (Ezaki et al., 2000, Ryan *et al.*, 2007, Sivaguru *et al.*, 2003a, Wang *et al.*, 2009). The details of how these genes function to confer Al^{3+} resistance are not well understood and it is not yet clear that they would be sufficiently effective to enhance the Al^{3+} resistance of crop species.

1.8 Objectives

A variety of biotechnological strategies have been used to increase the Al^{3+} resistance of plants and these have been summarized above. The most promising strategy to date has increased the efflux of malate anions from roots by over-expressing the *TaALMT1* gene from wheat. *TaALMT1* encodes an anion channel and has successfully been used to increase the Al^{3+} resistance of important crop plants such as wheat and barley (Delhaize et al 2004; Pereira et al 2010). One published study mentions a similar approach in crop plants using a *MATE* gene to increase citrate efflux. That single study described the over-expression of the sorghum *SbMATE* gene in wheat (Magalhaes et al 2007) but the phenotype observed in the T1 generation proved unstable in later generations (LV Kochian: personal communication). The objectives of this study are to determine whether over-expression of *MATE* genes can stably increase the Al^{3+} resistance of the important crop species barley by increasing citrate efflux from roots. The three *MATE* genes from barley (*HvAACT1*), Arabidopsis (*Frd3*) and sorghum (*SbMATE*) were expressed in barley under a constitutive promoter. The resulting plants were characterized for citrate efflux from roots and Al^{3+} resistance in hydroponic solution and in acid soil.

CHAPTER 2 General materials and methods

2.1 Transformation of *E. coli*

2.1.1 Preparation of *E. coli* cells for electroporation

A fresh colony of DH5α was inoculated in 5 ml of SOB (20 g tryptone, 5 g yeast extract, 0.5 mM NaCl, 2.5 ml 1M KCl in 1L, pH 7.0) medium in a 50 ml sterile conical tube. They were vigorously shaken at 180 rpm overnight at 37°C. 2.5 ml of cells from above were applied into 250 ml of SOB (without magnesium) in a 1 L flask. They were grown for 2 to 3 h with vigorous aeration at 37°C until OD_{550} of the cells reach 0.8. Cells were harvested by centrifugation at 5,000 rpm in a centrifuge for 10 min in sterile centrifuge tubes. The cell pellet was resuspended with a small amount of WB (10% glycerol), then ice cold WB was applied to the bottle and mixed gently. The cell suspension was centrifuged at 5,000 rpm for 15 min and then the supernatant was carefully poured off.

The pellet was washed a second time, and then resuspended to the final volume about 1 ml. Cells can be used immediately or frozen in 0.05 ml aliquots in freezer vials using liquid nitrogen and then stored frozen cells at -80°C.

2.1.2 Electroporation

An aliquot of competent cells were thawed on ice and then 1 µl of ligation reaction was added to the competent cells. They were mixed quickly and placed on ice for 10 min.

The 50 µl was transferred to the pre-cooled transformation cuvette. The cuvette was placed in the electroporator (Micropulser^{TM} BioRad, USA). The sample was electroporated at 2.4 kV discharge voltage. And then 2 ml of SOC medium was immediately added. The mixture was transferred to an Eppendorf tube and shaken for 30 min at 37 ^{o}C. The cells were placed on LB medium containing 100 µg/ml ampicillin, 40 µl of 20 mg/ml X-Gal (5-Bromo-4-chloro-Indoly-β-D-Galactoside), and 40 µl of 100 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside). The plates were placed at 37 ^{o}C for about 16 h.

2.2 Sequencing

BigDye terminator v3.0 cycle sequencing kit was used in the reaction. The reaction was as follows: 0.5 μl BigDye terminator, 3.5 μl 5×Buffer，2 μl 1.6 μM primer, and 25 ng of template times the length of the template in kilo bases, and they were made up to 20 μl. The tubes were placed in a PCR machine. The cycle sequencing program was as follows: 95 ^{o}C for 5 min, 50 cycles of 95 ^{o}C for 30 s, 55 ^{o}C for 10 s and 60 ^{o}C for 4 min. The solution was purified with BigDye terminator removal kit and dried down for sequencing by various service centres but usually by an ABI 3730 sequencer.

2.3 DNA isolation

2.3.1 SDS method for DNA extraction

2 ml Eppendorf tube with 100 mg tissue sample was frozen in liquid nitrogen. They were ground into powder with glass bar. 0.75 ml of extraction buffer (1.5% SDS, 0.1 M NaCl, 0.05 M EDTA, 0.1 M Tris-HCl, pH 8.5) was added to each sample, and then the samples were kept at 65°C for 30-60 min.

0.75 ml of phenol and chloroform (1:1) was added to each tube and vortex gently for 5 min. They were centrifuged at 13,200 rpm for 10 min. The supernatant was transferred into a new 1.5 ml tube containing 0.5 ml of cold isopropanol. They were mixed very well, and immediately centrifuged for 1 min at 13,200 rpm.

The supernatant was discarded. The DNA pallets were washed twice with 70% ethanol and then dissolved with 0.2 ml $TE_{0.1}$ containing 6 μl 10 mg/ml RNAse. They were kept at room temperature overnight. 1 μl of the DNA can be used for PCR.

2.3.2 Rapid DNA isolation method for PCR

About 2 cm leaf tip was ground in a 2 ml tube with 66 μl 0.15 M NaOH. After they were boiled at 98 ^{o}C for 1 min, 120 μl 0.1M Tris (pH8.0) was added to neutralize the solution. 1 μl of the solution (will probably require 10 times dilution with water) can be used for PCR.

2.4 Amplification from gDNA and plasmid DNA

The reaction was as follows: 5 μl HotStar^{TM} Master Mix (Qiagen), 1 μl Primer Mix (0.5 μl 10 μM forward primer and 0.5 μl 10 μM reverse primer), 1 μl DNA and 3 μl H_2O. The reaction was performed at 95 ^{o}C for 15 min, (95 ^{o}C for 30 s, 60 ^{o}C for 30 s, 72 ^{o}C for 1 min)×34 cycles, and 72^{o}C for 10 min.

2.5 Quantitative Real-Time PCR

Root apices (4-5 mm) were collected and the total RNA was extracted using RNeasy^{TM} Minikit (Qiagen). Contaminating DNA was removed by the inclusion of one DNase step and 1 μg of total total RNA, 1x RT buffer, 10 mM of each dNTP, 500 ng of oligo$(dT)_{15}$ primer, 0.2 M dithiothreitol (DTT) and 1 U of SuperScript II Reverse Transcriptase (Invitrogen) were used to prepare the cDNA. Reaction with 20 μl final volume were incubated at 25^{o}C for 5 min, and then at 42^{o}C for 60 min followed by a RNaseH degradation step performed for 30 min at 37^{o}C. Samples were run in a Rotor-Gene 3000 Real Time Cycler (Corbett Research, Australia) after the 10 μl reaction mixture containing 4.5 μl of cDNA diluted to 1:50, 5 μl of SYBR Green JumpStart Taq ReadyMix (Sigma) and 0.5 μl of primer mix (50:50 mix of forward and reverse primers at 10 pmol/μl each) was prepared. The primers used to normalize the transgene expression based on barley endogenous genes *Actin* were 7F and 7R (**Table 2.5**). The primers 6F and 6R target wheat reference gene *GAPDH* (**Table 2.5**). Primers used to measure expression of *SbMATE* were 2F and 2R (**Table 2.5**); for *HvAACT1* were 3F and 3R; and for *frd3* were 4F and 4R (see **Table 2.5**). Cycling conditions were 5 min at 94^{o}C, followed by 45 cycles at 94^{o}C for 15 sec, 55^{o}C for 15 sec, and 72^{o}C for 20 sec. At the end, a melting curve of the amplified fragments was produced by increasing the temperature every 1^{o}C from 55 to 99^{o}C.

2.6 Measurements of citrate efflux from root apices

Citrate efflux from excised root apices was measured according to Wang et al. (2007) with some modifications. After 4 days growing in the nutrient solution (without added $AlCl_3$) described as bellow, root apices (5 mm) were excised from seedlings of the same line and washed for 20 min on a platform shaker (60 rpm) in 5 ml sterilized glass vials containing 1 ml of control solution (0.2 mM $CaCl_2$, pH

4.3). After that the control solution was discarded, 1 ml of treatment solution (with or without $AlCl_3$) was added and the tubes containing the root tips were shaken (60 rpm) for 2 h. For citrate measurements, samples were dried on a rotary vacuum drier and resuspended in 114 µl assay solution consisting of 100 mM Tris, pH 8.0, 0.25 µl malate dehydrogenase (M-7383, Sigma), 0.25 µl lactate dehydrogenase (107042, Roche), and 14 µl NADH (prepared by dissolving 16 mg NADH and 15 mg $NaHCO_3$ in 2 ml water). NADH consumption, read as the decrease in A_{340}, was monitored after the reaction was initiated with 1 µl citrate lyase (C0897, Sigma, prepared by dissolving 5 mg citrate lyase in 100 µl H_2O) on a Cary 50 Bio Spectrophotometer. The initial citrate content in each sample was calculated from the standard curve.

2.7 Measurements of malate efflux from root apices

Malate efflux from excised root apices was measured according to Pereira et al. (2010) with some modifications. After 4 days growing in the nutrient solution (without $AlCl_3$) described as bellow, eight root apices (4 mm) were excised from seedlings of the same line and washed for 20 min on a platform shaker (60 rpm) in 5 ml sterilized glass vials containing 1 ml of control solution (0.2 mM $CaCl_2$, pH 4.3). After that the control solution was discarded, 1 ml of treatment solution (with or without Al) was added and the tubes containing the root tips were shaken (60 rpm) for 2 h. For malate measurement, 0.75 ml buffer (prepared by dissolving 34.2 g Glycine and 75 ml Hydrazine in 0.9 L water) and 40 µl NAD (N-0632, Sigma, dissolving 30 mg NAD in 1 ml H_2O) were added to 0.7 ml samples. NAD consumption, read as the increase in A_{340}, was monitored after the reaction was initiated by adding 3 µl MDH (M-7383, Sigma) on a Cary 50 Bio Spectrophotometer. The initial malate content in each sample was calculated according to the standard curve.

2.8 Measurements of Al^{3+} resistance in hydroponic culture

Seed were sterilized with 20% bleach (White King^{TM}) for 15 min, rinsed thoroughly and germinated in the dark for 2 days at 4^{o}C and then 2 days at 28^{o}C. A nutrient solution, with pH adjusted to 4.3, containing 500 µM KNO_3, 500 µM $CaCl_2$, 500 µM NH_4NO_3, 150 µM $MgSO_4$, 100 µM KH_2PO_4, 2 µM Fe:EDTA, 11 µM H_3BO_3, 2

μM $MnCl_2$, 0.35 μM $ZnCl_2$ and 0.2 μM $CuCl_2$ was prepared. For barley Al^{3+} resistance experiments, the final $AlCl_3$ concentrations of 0, 1, 2 and 4 μM were used in the hydroponic solutions. The initial length of the longest root was measured before the experiments. After 4 days growing in nutrient solutions, the seedlings were removed and the length of the longest root was measured again. The RRG was estimated as: (net root growth in Al^{3+} treatment/net root growth in control solution). Standard errors of the relative root growth (SE_{RRG}) were calculated as follows:

$SE_{RRG} = RRG \times [(SE_x/x)^2 + (SE_y/y)^2]^{1/2}$, where x and y represent the mean net root length in the control treatment and Al^{3+} treatment, and SE_x and SE_y represent the standard errors of those means.

2.9 Measurements of Al^{3+} resistance in soil

An acidic red ferrosol was obtained from the Robertson region of New South Wales, Australia (34° 35's, 150° 36'E). The pH of 0.01 M $CaCl_2$ extract was 4.33 and the exchangeable Al^{3+} was 21% of the total cations. The limed soil was treated by adding a rate of 5 g lime kg^{-1} dry soil to raise the pH to 5.18 and reduce the soluble Al^{3+} to below 1%. The soil was wetted to 90% field capacity. 1.3 kg of soils was added into the deep pots (diameter of 9 cm and height of 22 cm). Seed were sterilized and germinated as described above. For each line, twelve seedlings with similar root length were planted in three acid pots and three limed pots, 2 seedlings in each pot. About 6 days later, the seedlings were harvested. The fresh shoot weight, fresh root weight, the longest root length, the second longest root length, the total root length were measured. Among them, the total root including the lateral roots were scanned from WinRHIZO™, and the total root length was calculated by the software. The RRG and standard errors were calculated as above.

2.10 Triparental mating to introduce binary plasmids into *Agrobacterium*

Triparental mating is used for the transfer of DNA from *E.coli* to *Agrobacterium* before transfer into plant cells. This required an *E.coli* carrying the plasmid of interest, the *E.coli* carrying the helper plasmid pRK2013 (with kanamycin resistance gene) and *Agrobacterium* strain AGL1 (with rifampicin resistance gene) were used in the experiment. The *Agrobacterium* was streaked out onto LBMG (1.5%

agar in MGL) (**Table 2.1**) plate containing rifampicin at 20 μg/ml and grown for 2 days at 28 ^{o}C. The next day, a plate of *E.coli* carrying the helper plasmid pRK2013 was streaked onto LB agar with kanamycin at 75 μg/ml and a plate of *E.coli* carrying the recombinant plasmid (*pWBvec8:HvAACT1*, *pWBvec8:SbMATE* or *pWBvec8:Frd3* all with the spectinomycin resistance gene) was streaked onto LB agar with spectinomycin at 50 μg/ml. These two plates were incubated at 37 ^{o}C overnight. On the third day, a loopful of each culture was mixed together on the center of a LBMG agar plate with no antibiotics and incubated at 28 ^{o}C for 2 days. In addition, the pRK2013 *E.coli* and the *Agrobacterium* only were mixed as a control. Some of the mixture was streaked onto a LBMG agar plate with rifampicin at 20 μg/ml and spectinomycin at 50 μg/ml two days later. Single colonies appeared. A single colony of *Agrobacterium* was put into 10 ml MGL medium with 50 μg/ml spectinomycin and 20 μg/ml rifampicin. The culture was shaken at 27 ^{o}C for 40 h in darkness. To ensure the *Agrobacterium* has taken up the intact binary construct and no rearrangement of the gene occurred, the plasmid was isolated from *Agrobacterium* and sequenced. Because the concentration of plasmid isolated from *Agrobacterium* was too low, the plasmid was transformed into *E.coli* and isolated to do sequencing. 125 μl of culture and 125μl of 30% aqueous glycerol were mixed in an Eppendorf tube. They were kept at room temperature for at least 6 h before they were stored at -80 ^{o}C to make standard inoculums. 100×macroelements (FHG without phytagel) (**Table 2.2**)：16.5 g/L NH_4NO_3, 190 g/L KNO_3, 44 g/L $CaCl_2.2H_2O$, 37 g/L $MgSO_4.7H_2O$ and 17 g/L KH_2PO_4.

Table 2.1 MGL medium

Ingredients	Weight
Mannitol	5.0 g
L-glutamic acid	1.0 g
KH_2PO_4	0.25 g
NaCl	0.1 g
$MgSO_4 \cdot 7H_2O$	0.1 g
Trypton	5 g
Yeast extract	2.5 g
Biotin	1 μg
Total	1 L with H_2O (pH 7.0)

Table 2.2 FHG medium

Ingredients	Weight or volume
100×macroelements (FHG)	10 ml
200×MS Micro-nutrient	5 ml
MS iron/EDTA (10 mM)	10ml
Myo-inositol	0.10 g
Glutamine	0.73 g
Maltose	62 g
Total	1 L with H_2O

(pH 5.7, add 3.0 g phytagel. After autocalving, 1 ml 1mg/ml thiamine HCl, 1.0 ml 200 mg/ml timentin and 0.4 ml 50 mg/ml hygromycin.)

2.11 Barley transformation

Barley transformation experiments were performed as described by Tingay et al. (1997) and briefly outlined below. Plants of spring barley cultivar, Golden Promise (GP), were grown in a growth cabinet at 18 ^{o}C for 16 h light period and 13 ^{o}C for 8 h dark period. Plants were maintained in a healthy, pest and stress free conditions.

2.11.1 Isolation of barley embryos

Spikes of barley were harvested when the immature embryos were 1.5-2.5 mm in length. The seed were removed from the spikelets and then sterilized with a brief rinse with 70% ethanol and 8 min of bleach (finally 0.8% available chlorine). The seed were drained off and thoroughly rinsed with sterile water. Embryos were excised squeezed out from seed under microscope and the embryonic axis was removed with a sharp scalpel blade. 50 explants were placed scutellum-side up on BCI medium without timentin and hygromycin.

2.11.2 Growth of *Agrobacterium*

250 µl standard inoculums was inoculated into 5 ml MGL medium without selection agents, and shaken for 24 h at 27 ^{o}C. When the OD_{550} of the culture reached 0.5 to 1.0, it was used for barley transformation.

2.11.3 Innoculation of the embryos

150 µl suspensions of *Agrobacterium* were applied to the explants on BCI medium (**Table 2.3**). The embryos were flipped over so the cut surface was up. The scutellums were transferred to fresh BCI medium (without selection agents) with cut side up. 25 embryos were placed evenly on the plate. They were grown at 24 ^{o}C for 2-3 days in dark. 3 days later, these embryos were transferred to BCI medium with 50 µg/ml hygromycin and 150 µg/ml timentin. After 2 weeks, healthy callus came out. The callus was transferred to BCI medium with 50 µg/ml hygromycin and 150 µg/ml timentin every two weeks.

After 6 weeks, the calluses were transferred to the regeneration medium FHG in light at 24 ^{o}C every two weeks. The shoots began appear after the calli were grown in FHG for 4-6 weeks.

Table 2.3 BCI medium

Ingredients	Weight or volume
20×MS macro-nutrient	50 ml
200×MS Micro-nutrient	5 ml
MS iron/EDTA (10 mM)	10ml
Myo-inositol	0.25 g
Casein Hydrolysate (N-Z case TT)	1.0 g
L-Proline	0.69 g
Maltose	30 g
Total	1 L with H_2O

(pH to 5.9, add 3.5 g phytagel. After autocalving, 1 ml 1mg/ml thiamine HCl, 2.5 ml 1mg/ml dicamba and 0.75 ml 200 mg/ml timentin and 1ml 50mg/ml hygromycin.)

Table 2.4 RM medium

Ingredients	Weight or volume
20×MS macro-nutrient	50 ml
200×MS Micro-nutrient	5 ml
MS iron/EDTA (10 mM)	10ml
Casein Hydrolysate (N-Z case TT)	1.0 g
Myo-inositol	0.25 g
L-proline	0.69 g
Maltose	30 g
Total	1 L with H_2O

(pH to 5.9, add 3.0 g phytagel. After autocalving, 0.4 ml 1mg/ml thiamine HCl and 1 ml 50 mg/ml hygromycin.)

As shoots outgrew petri dish, they were transferred to rooting medium (RM) (**Table 2.4**). After there were several strong white roots, the plants were transferred to nutrient solution or to soil and grown in the glasshouse.

2.11.4 Analysis of transgenic plants

T0 plants were analyzed for the presence of introduced genes using the following primers (see **Table 2.5**): (*i*) 8F and 8R for the *SbMATE* gene: GTCACCACGTCGTTCGTC and GGGTGCAGATCTGGAAGG; (*ii*) 5F and 5R for the *HvAACT1* gene: TGCAGCATCTATTCATATGC and AGAGGTAGAGCCCCGTCGT respectively; and (*iii*) 4F and 4R for the *frd3* gene: GCCCATGTCATTTCTCAGTACTTCA and TTCCAAACTGCAAATCCCCGAAG respectively. Positive T0 plants were grown until T2 generation, and some homozygous and null lines were recovered for each gene. Seed from T1 and T2 generation were used to analyse expression of transgenes, citrate efflux from the roots and the relative root growth in hydroponic cultures and acid soil.

2.11.5 System for naming transgenic materials

Transgenic plants from tissue culture are called T0. The seed harvested from T0 lines were called T1 seed. Several individual T1 seed were grown, and each plant is named with (gene_T1_line): the gene name followed by the generation name, and finally the line number. T1 plants segregate for the transgene. They either be homozygous for the transgene, hemizygous for the transgene or null segregants (no transgene). The ratio of these depends on the number of transgene inserts. For a single transgene insert the T1 generation segregate as 1:2:1 (homozygous, hemizygous and null respectively) (**Figure 2.1**). The T2 and T3 lines are derived from seed collected from individual plants. For the later generation 'Null' was placed in front of the null lines to make the data in the figures more easily interpreted.

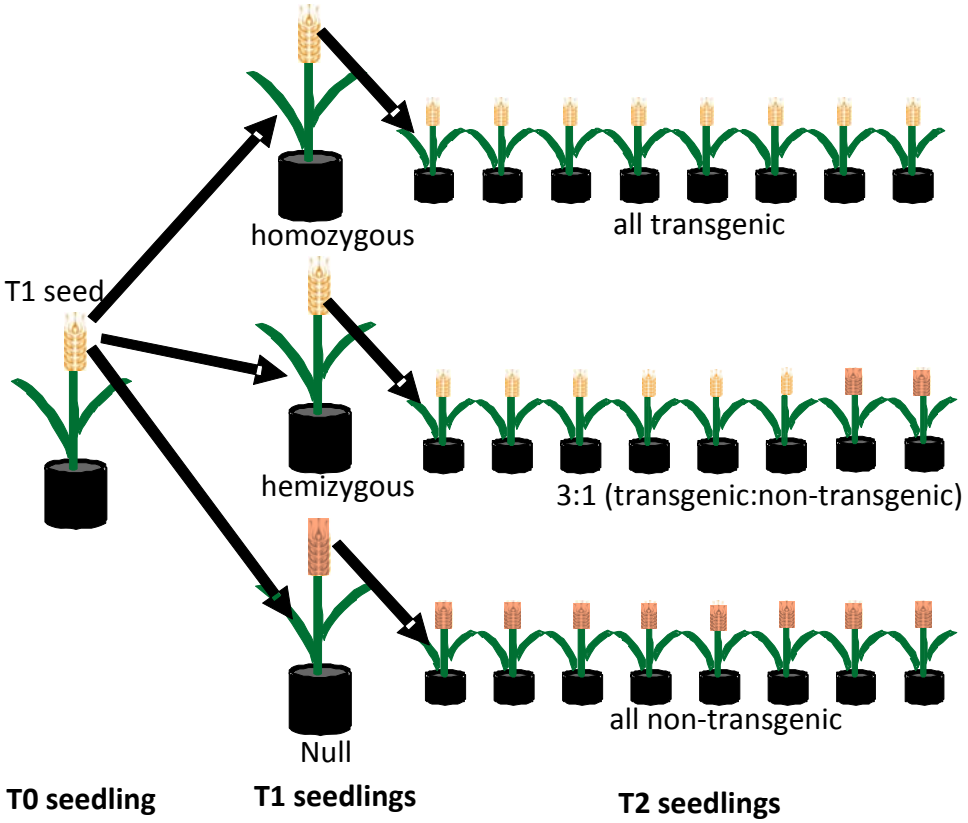


Figure 2.1 Nomenclature of the transgenic plants. For a single transgene insert, the segregation ratio of transgenic and non-transgenic plants in the T1 generation is 3:1. All of the T2 seedlings from homozygous T1 are transgenic; T2 seedlings from hemizygous T1have 3:1 segregation ratio (transgenic:non-transgenic); all of the T2 seedlings from null T1 are non-transgenic. Yellow spikelet represents transgenic and pink spikelet represents non-transgenic.)

2.12 Leaf assay

A very simple leaf assay method was used to discriminate transgenic and non-transgenic barley as described below. The transgenic plants transformed with vector containing antibiotic gene hygromycin were sterilized with bleach (0.8% available chorine) and then grown in petri dishes for one week. One homozygous transgenic barley line *TaALMT1* (Delhaize et al., 2004) was a positive control in all leaf assay experiments.

One cm leaf tips were cut from plants seedlings and embedded in solid RM medium (**Table 2.4**) containing 200mg/L hygromycin. They were placed in the culture room (24 ^{o}C) with 16 h light and 8 h dark. Non-transgenic barley plants had noticeable symptoms of either bleaching or necrosis after four days on the medium and were completely bleached or necrotic after one week. Transgenic leaf tips remained green and healthy over this period.

Table 2.5 Primers used in the experiments

Serial No.	Purpose	Primer sequences (from 5' to 3')	Note
1F	pWUbi construct Sequencing Forward	CTTGATATACTTGGATGATGG	Forward primer in the ADH intron
1R	pWUbi construct Sequencing Backward	GTGTTCTAAGCTAGCCTGG	Reverse primer in the terminator-pWUbi tml
2F	*SbMATE* expression Forward	ACCTGATAACGCTGATAATGCTGAG	
2R	*SbMATE* expression Backward	CAGCAGAAGGAATCCGCATCC	
3F	*HvAACT1* expression Forward	AGCAGCCAAGACCTTGAGAA	
3R	*HvAACT1* expression Backward	GCCGAAAAGATCAGGAACAC	
4F	*Frd3* expression Forward	GCCCATGTCATTTCTCAGTACTTCA	
4R	*Frd3* expression Backward	TTCCAAACTGCAAATCCCCGAAG	
5F	*HvAACT1* transgenic screening Forward	TGCAGCATCTATTCATATGC	Ubiquitin intron
5R	*HvAACT1* transgenic screening Backward	AGAGGTAGAGCCCCGTCGT	Inside *HvAACT1* gene
6F	Wheat endogenous gene GAPDH Forward	TCAGACTCCTCCTTGATAGC	
6R	Wheat endogenous gene GAPDH Backward	GTTGAGGGTTTGATGACCAC	
7F	Barley Actin gene Forward	GACTCTGGTGATGGTGTCAGC	
7R	Barley Actin gene Backward	GGCTGGAAGAGGACCTCAGG	
8F	*SbMATE* forward for checking insert	GTCACCACGTCGTTCGTC	
8R	*SbMATE* reverse for checking insert	GGGTGCAGATCTGGAAGG	

CHAPTER 3 Over-expression of *HvAACT1* in barley

3.1 Introduction

The genetic variations in barley for Al^{3+} resistance is small compared to other cereals but it is sufficient to select for in traditional breeding strategies (Minella and Sorrells, 1992). Foy (1996) screened ten barley cultivars for Al^{3+} resistance by growing them in pots of acid subsoil, and he found Tennessee Winter 52, Volla, Dayton and Herta were significantly more resistant to the acid. Moroni et al. (2010) found that Brindabella was the most resistant genotype. Ma et al.(1997b) used a staining method to screen 600 barley lines from which they selected 90 lines showing intermediate Al^{3+} resistance compared to Al^{3+}-resistant wheat.

To date the only resistance mechanism characterized in barley relies on citrate efflux (Zhao *et al.*, 2003). The secretion of citrate from barley roots is rapid, activated by Al^{3+} and temperature-dependent (less at low temperatures). Citrate secretion and Al^{3+} resistance are positively correlated in 21 barley cultivars differing in Al^{3+} resistance (Zhao et al., 2003). These results indicate that secretion of citrate is a major mechanism involved in Al^{3+} resistance in barley. The citrate is thought to protect plants from Al^{3+} toxicity by binding to the harmful Al^{3+} cations in the apoplasm and detoxifying them.

Furukawa et al. (2007) cloned the Al^{3+} resistant gene in barley using a map-based cloning approach. New markers were developed between Bmac310 and Bmag353 based on EST information and the synteny with rice. The syntenous region in the rice genome was restricted to approximately 140kb containing 21 annotated gene models (Furukawa et al., 2007). A microarray analysis was performed to identify related genes in barley that were more highly expressed in Murasakimochi (Al^{3+}-resistant) than Morex (Al^{3+}-sensitive) in the presence or absence of Al^{3+} treatment. By comparing the expression of 25 ESTs derived from genes located between the markers Bmac310 and Bmag353, only one gene named *HvAACT1* (aluminium activated citrate transporter) encoding a member of the multidrug and toxic compound extrusion (MATE) family was expressed by over 20-fold higher in Murasakimochi compared to Morex. Similar to *TaALMT1* in wheat, expression of

HvAACT1 in the root apices is constitutive and not affected by Al^{3+} treatment (Furukawa et al., 2007). The coding region of the gene is 1668 bp resulting in a deduced protein of 555 amino acids which consists of 12 introns and 13 exons. There are only two nucleotides and one amino acid differences between the parental lines Murasakimochi and Morex. However, these SNPs cannot explain the differences in *HvAACT1* expression and Al^{3+} resistance since expression of the *HvAACT1* cRNA from either parent in *Xenopus laevis* oocytes generates the same Al^{3+}-activated citrate efflux (Furukawa et al., 2007). This suggests that the expression level of *HvAACT1* determines the level of Al^{3+} resistance and not the protein sequence. This was confirmed by showing there were positive correlations between the level of *HvAACT1* expression, the Al^{3+}-induced citrated secretion and Al^{3+} resistance among a range of barley genotypes. *HvAACT1* is expressed in both roots and shoots, but the level of expression was higher in the roots than in the shoots (Furukawa et al., 2007).

The function of *HvAACT1* was examined further in a transgenic tobacco plant. Greater Al^{3+}-activated citrate efflux and Al^{3+} resistance was observed in transgenic tobacco compared to non-transgenic control plants (Furukawa et al., 2007). *HvAACT1* is expressed in the epidermal cells of the barley root tips based on *in situ* hybridization analysis with HvAACT1 antibody. Subcellular localization of HvAACT1 fused to GFP in onion epidermal cells revealed that HvAACT1 is located on the plasma membrane of the cells.

The aim of this Chapter was to determine whether over-expression of the *HvAACT1* gene in barley can enhance Al^{3+} resistance.

3.2 Materials and methods

HvAACT1 cDNA in the pTA2 plasmid was kindly provided by Professor Jianfeng Ma (Okayama University, Japan) (Furukawa et al., 2007). The cDNA was introduced into pWBvec8 and driven by the maize ubiquitin promoter. The process of constructing the vector is described below and summarized in **Figure S1**.

The pWubi tml was first digested with *Not*I, and the 2415 bp fragment containing the ubiquitin promoter, a maize ubiquitin intron and the *tml* terminator sequence was excised and purified from a 1% agarose gel. The fragment was introduced into

the *Not*I site of the binary vector pWBvec8 resulting in two types of constructs with different orientations of the inserted fragment. The construct with the band sizes of 2099bp and 11771bp after digestion with *Bam*HI was selected (**Figure S2**) and sequenced.

The *HvAACT1* cDNA was excised from pTA2 with *Eco*RI and introduced into pBluescript II. The correct plasmids were identified by digestion with *PstI*. The plasmid with the insert in correct orientation was used for vector construction (**Figure S3**). To check the insert size and orientation, the plasmids were digested with *Eco*RI and *Pst*I respectively. *Eco*RI was used to check the size of the correct insert, which was about 1.7 kb after digestion and *Pst*I was used to identify the orientation of the insert. After they were digested with *Pst*I, the sizes of the bands were 0.4 kb and 4.3 kb or 1.3 kb and 3.4 kb. The correct orientation produced bands of 1.3 kb and 3.4 kb and this plasmid was digested with *Sma*I and *Kpn*I to obtain the 1.7 kb *HvAACT1* fragment. This fragment was purified, ligated into the binary vector pWBvec8:ubiquitin and the resulting construct transformed into *E.coli* DH5α.

Positive colonies were detected by PCR using primers 5F and 5R, which amplify the ubiquitin and HvAACT1 regions (**Table 2.5**). The selected colony was further analyzed by digestion with *Bam*HI or by double digestion with *Sma*I and *Kpn*I. The correct construct generated fragments of 1.7 and 12.2 kb when double-digested with *Sma*I and *Kpn*I (**Figure S4**). The full construct was sequenced before being introduced into *Agrobacterium* by triparental mating (see chapter 2). The plasmid was then extracted from the *Agrobacterium* and sequenced to make sure it was correct.

Agrobacterium transformed with the pWBvec8:ubiquitin:HvAACT1 vector was used to inoculate immature embryos of the barley cultivar Golden Promise. Wild-type Golden Promise was used as a negative control in subsequent characterizations. The Al^{3+}-resistant controls included Dayton, one of the most Al^{3+} resistant barley genotypes, and a transgenic barley line over-expressing the wheat *TaALMT1* gene (Delhaize et al., 2004). In some experiments the Al^{3+}-resistant wheat cultivar Carazinho was included because it possesses Al^{3+}-dependent malate efflux which is absent in barley.

The methods for plant growth, Al^{3+} resistance measurements in hydroponic solutions and soils, citrate and malate assays and RNA expression are described in Chapter 2.

3.3 Results

3.3.1 Barley transformation

Agrobacterium carrying the binary vector was used to transform barley embryos. At the tissue culture stage, 179 calli developed from the 395 embryos, and were inoculated to generate a total of 119 transgenic events. Every embryo generated from 1 to over 10 regenerated plants. Using the primers 5F and 5R (see **Table 2.5**), PCR showed that all 119 plantlets carried the *HvAACT1* transgene (**Figure S4**). These primers do not amplify the endogenous *HvAACT1* gene because the forward primer targets the maize ubiquitin intron in the construct. The transformation efficiency was about 30%.

3.3.2 Analysis of T0 *HvAACT1* barley plants

13 T0 plants were selected randomly and transferred from tissue culture to hydroponic culture. Citrate efflux from root apices was measured and most of the transgenic lines had greater citrate efflux than the wild-type cultivar Golden Promise (**Figure 3.1a**). The root mass on these plants was too small to provide biological replicates. All 119 of the T0 plants were transferred to soil to generate T1 seed.

3.3.3 Characterization of T1 *HvAACT1* barley plants

Since T1 plants segregate for the transgene among the individual plants, assuming they harbor a single insert, would be either homozygous, hemizygous, or nulls segregants for the *HvAACT1* transgene in a ratio of 1:2:1. Null plants in each line were detected by the leaf assay for antibiotic resistance (see **Chapter 2**). Only seedlings resistant to hygromycin (either homozygous or hemizygous) were used in the citrate efflux and Al^{3+}-resistance measurements. Seed from eight of the T1 lines were germinated and grown in hydroponics for citrate efflux assays. Among these transgenic lines, two lines, T1_17A and T1_33A, had the highest citrate efflux when compared to Golden Promise and were selected to generate T2 lines (**Figure 3.1b**).

Additional two T1 lines, T1_51 and T1_52B, were selected after another 17 T1 lines (selected randomly) were screened for Al^{3+} resistance in hydroponics containing 1 μM Al^{3+} (**Figure 3.2**). T1_73 also performed well in this Al^{3+}-resistance assay but the seed from this line was limited. Therefore 12 seedlings of each of these T1 lines (17A, 33A, 51 and 52B) were grown in the glasshouse to generate T2 seed.

These T1 lines were also checked for segregation of the *HvAACT1* transgene using PCR with the primers 5F and 5R. The results indicated that T1_51 segregated with a 3:1 ratio (transgenic to non-transgenic) and therefore is likely to contain single transgene insertion. Lines T1_17A and T1_33A segregated with an 11:1 ratio which may indicate two transgene insertions (χ^2 tested at α=0.05 level). No null lines were detected among the twelve T1 seedlings of T1_52B which may mean that it contains multiple insertions. A larger number of T1 seedlings probably need to be screened to increase the confidence in these segregation ratios.

Table 3.1 Segregation analysis of T2 *HvAACT1* lines determined by the leaf assay for antibiotic resistance

T2 Lines	Resistant	Sensitive	Total
T2_17A			
1	9	0	9
2	11	0	11
3	14	0	14
4	9	0	9
5	2	1	3
6	5	0	5
7	1	0	1
8	5	3	8
9	0	8	8
10	10	4	14
11	4	0	4
12	7	4	11
T2_33A			
1	15	0	15
2	10	6	16
3	7	2	9
4	0	3	3
5	8	2	10
6	15	3	18
7	12	3	15
8	12	0	12
9	14	4	18
10	13	0	13
11	3	0	3
12	3	0	3
T2_51			
1	0	10	10
2	15	3	18
3	14	0	14
4	16	0	16
5	0	1	1
6	3	2	5
7	4	2	6
8	10	0	10
9	10	2	12
10	0	12	12
11	5	7	12
12	10	7	17
T2_52B			
1	7	1	8
2	4	0	4
3	7	3	10
4	12	0	12
5	5	0	5
6	5	3	8
7	23	4	27
8	20	1	21
9	12	2	14
10	11	4	15
11	24	0	24
12	18	0	18

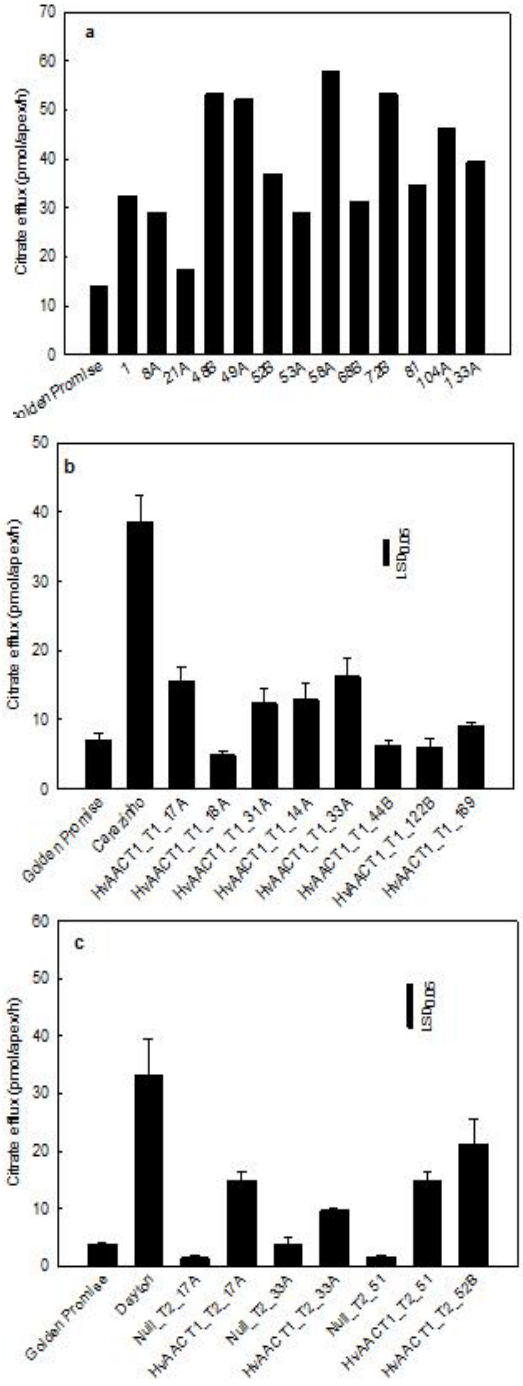


Figure 3.1 Citrate efflux from root apices of transgenic and various control plants including the parental barley, Golden Promise, the Al^{3+}-resistant barley Dayton and the Al^{3+}-resistant wheat Carazinho. a. Citrate efflux in the T0 *HvAACT1* transgenic lines. Citrate efflux was measured from excised root apices of selected plants carrying *HvAACT1* in the presence of 50 μM $AlCl_3$. Data show the mean of citrate efflux. b. Citrate efflux in the T1 *HvAACT1* transgenic lines. Citrate efflux in 50 μM $AlCl_3$ was measured from eight root apices excised from two to three plants. Data show the mean citrate efflux and standard error (n=3). c. Citrate efflux in the T2 homozygous *HvAACT1* transgenic lines. Citrate efflux was measured from eight excised root apices with 50 μM $AlCl_3$ treatment. Data show the mean citrate efflux and standard error (n=4). $LSD_{0.05}$ are shown in the bars.

3.3.4 Characterization of T2 *HvAACT1* barley plants

Leaves from individual seedlings of the resulting T2 families were also assayed for resistance to hygromycin. However, the germination for many lines was poor and it was difficult to confirm the segregation ratios (**Table 3.1**). Nevertheless, for most transgenic events null and putative homozygous T2 lines could still be selected. The T2 transgenic lines chosen for future experimental use were T2_17A-3, T2_33A-1, T2_51-4 and T2_52B-11. For simplicity they were renamed as HvAACT1_T2_17A, HvAACT1_T2_33A, HvAACT1_T2_51 and HvAACT1_T2_52B. Null lines chosen for further experimentation were renamed Null_T2_17A, Null_T2_33A and Null_T2_51.

Assay for efflux of organic anions

Citrate efflux from root tips of T2 transgenic *HvAACT1* lines in the presence of 50 μM Al^{3+} was about four times greater than the wild-type Golden Promise and about half of the efflux from Dayton the Al^{3+}-resistant cultivar used as a positive control. All of the null lines were similar to Golden Promise, and a proportion of the low levels of citrate efflux from these lines (**Figure 3.1c**) could have been leakage from the cut surface of the root.

Al^{3+}-activated malate efflux from root apices was not observed in the homozygous lines, null lines or Golden Promise. However malate efflux was detected in a positive control, the Al^{3+}-resistant wheat cultivar Carazinho (**Figure 3.3**).

Expression of *HvAACT1*

Among the transgenics, line HvAACT1_T2_52B had the highest level of *HvAACT1* expression, which was about 3-fold greater than Dayton expressing the endogenous *HvAACT1* gene. Lines T2_17A and T2_33A had similar *HvAACT1* expression levels to Dayton, whereas line T1_51 had about one-third of the expression of Dayton (**Figure 3.4**). These differences in expression level may reflect the number of transgenes in these plants. We had already established above that the T1_51 was likely to have a single insert whereas the other lines were more likely to contain two or more copies. As expected, no expression was detected in the null lines or in Golden Promise.

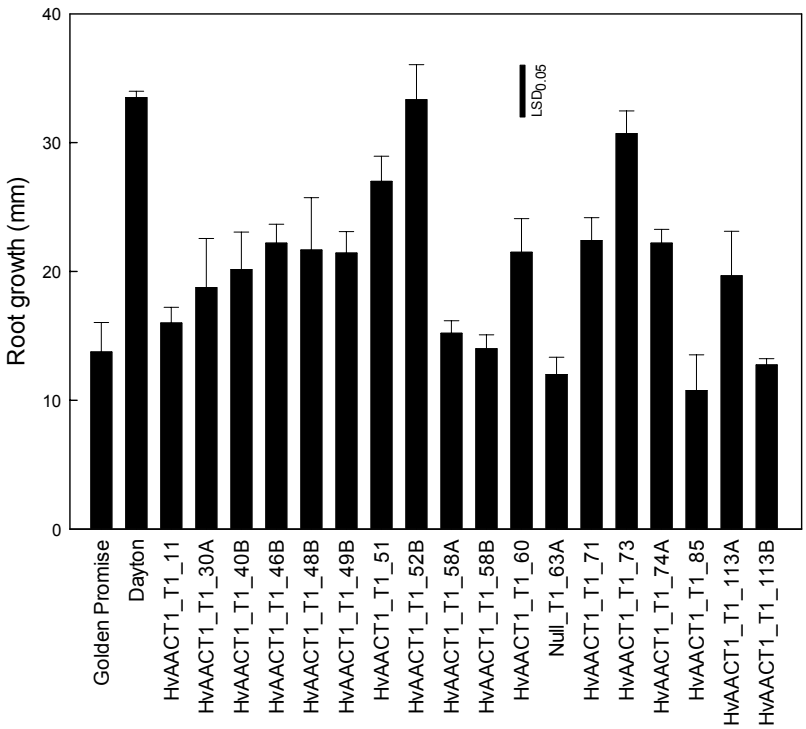


Figure 3.2 Root growth of T1 *HvAACT1* barley lines in hydroponic solution 1 μM $AlCl_3$. Root growth was measured before placing seed in hydoponics and after 4 days in nutrient solution. Data show the mean of root growth and standard error (n=7). The bar represents the $LSD_{0.05}$.

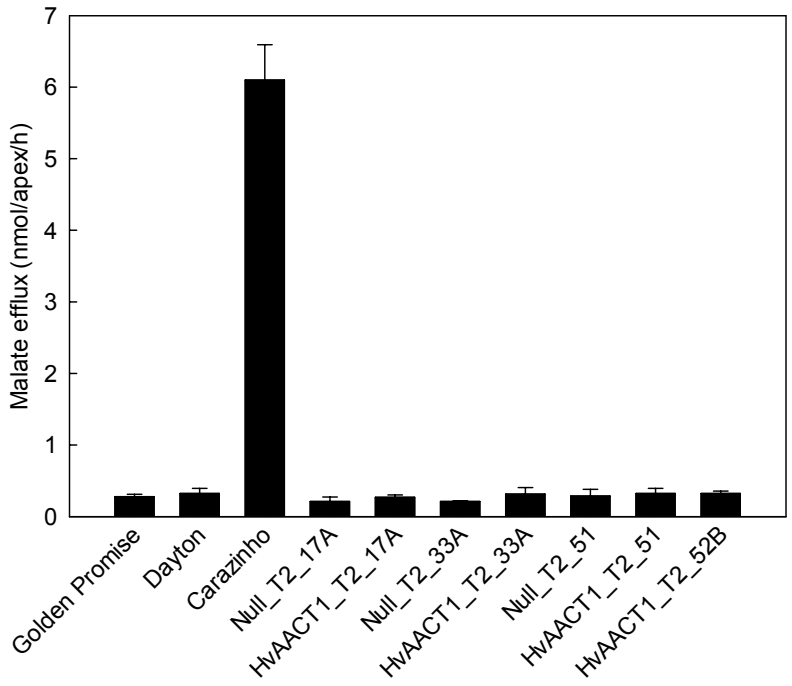


Figure 3.3 Malate efflux from root apices of T2 transgenic lines homozygous for *HvAACT1* and control plants. Malate efflux was measured from about eight excised root apices in the presence of 50 μM $AlCl_3$. Data show the mean of malate efflux (pmol/root/h) and standard error (n=4).

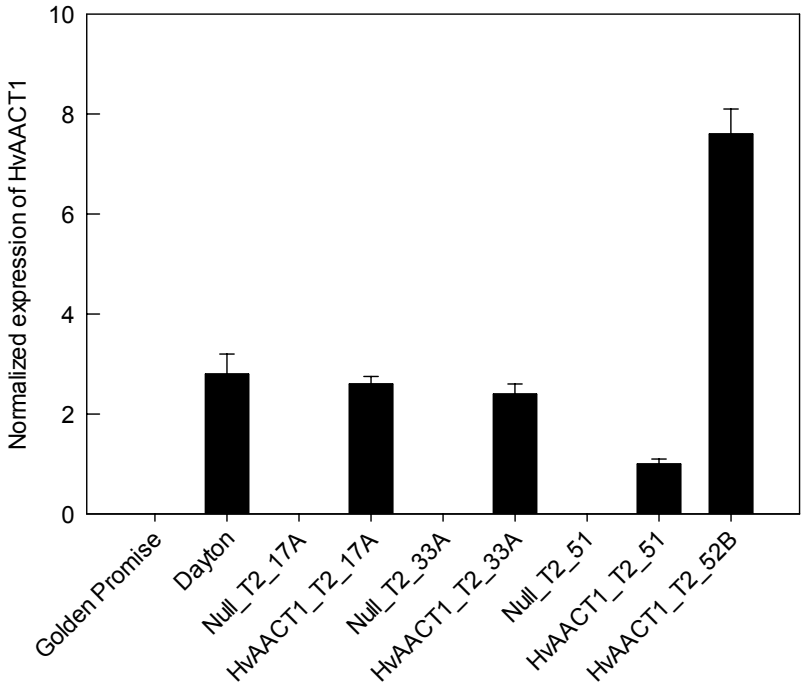


Figure 3.4 *HvAACT1* expression in transgenic T2 lines homozygous for *HvAACT1* and controls.Total RNA was extracted from eight root apices. Primer 3F and 3R (see **Table 2.5**) were used to amplify *HvAACT1* from transgene and endogenous expression. Primers 7F and 7R (see **Table 2.5**) were used to amplify the internal reference transcript from the *actin* gene. Data show the mean of normalized expression level compared to actin and standard error from two biological replicates each with two technical replicates.

Relative root growth: hydroponics experiments

Al^{3+} resistance in the transgenic lines was evaluated in hydroponic solutions containing 0, 1, 2 and 4 μM Al^{3+}. The root growth of all of the lines including the null lines ranged from 54 to 68 mm when grown in 0 μM Al^{3+} solutions. In 1 μM Al^{3+} solution, the root growth of the null lines (Null_T2_17A, Null_T2_33A and Null_T2_51) and the wild-type Golden Promise was inhibited by about 40%, while the transgenic lines (HvAACT1_T2_17A, HvAACT1_T2_33A, HvAACT1_T2_51 and HvAACT1_T2_52B) were not affected. The root growth of null lines and Golden Promise were significantly inhibited in 2 μM and 4 μM Al^{3+} solutions, whereas the root growth of transgenic lines expressing *HvAACT1* were approximately two-fold greater than their corresponding null lines (**Figure 3.5a**). The relative root growth (RRG) of these transgenic lines reached 110% in 1 μM Al^{3+}, which was similar to Dayton and approximately 80% higher than Golden Promise and the null lines. In 2 and 4 μM Al^{3+} solutions, the RRG of transgenic lines were 40-60% which was two-fold greater than Golden Promise and null lines but generally less than that of Dayton (**Figure 3.5b**).

Relative root growth: soil experiments

Plant growth was also conducted in an acid soil with a pH of 4.3-4.5 (soil extracted with 10 mM $CaCl_2$; Chapter 2). Soil was either unamended (acid) or limed to raise the pH to 5.18 which reduced the Al^{3+} toxicity of the soil. Germinated seed were planted in pots in a glasshouse and the following measurements were made after six-day growth: shoot fresh weight, root fresh weight, the longest root length, the second longest root length, the total root length and the distribution of root thicknesses. Four T2 transgenic lines, three null lines and the wild-type control cultivars Golden Promise and Dayton were included in these experiments. No significant difference in relative shoot fresh weight was detected between transgenic lines and their null simster lines (**Figure 3.6b**). Since significant differences in root fresh weight were detected among these lines in limed soil, relative root weight was a more meaningful comparison (**Figure 3.7**). Relative root weight also showed a

significant difference between the transgenic lines and null lines. The relative root weight for the transgenic lines was about 1.5 to 2-fold greater than the null lines and Golden Promise. The HvAACT1_T2_17A line exhibiting the highest relative root fresh weight was similar to the Al^{3+}-resistance cultivar Dayton (**Figure 3.7b**).

In limed soil the average length of the longest root ranged from 170 to 240 mm (**Figure 3.8a**). In acid soil, Dayton showed no reduction in the length of its longest root, while the transgenic lines showed a 40% reduction, and the null lines and Golden Promise showed a 50% to 70% reduction. A similar result was obtained for the second longest root. Dayton showed the highest relative longest root length and second longest root lengths with around 100% (**Figure 3.9)**. In general root growth in the four transgenic lines over-expressing *HvAACT1* was less inhibited by acid soil than the null lines and the parental cultivar Golden Promise. Among the four transgenic lines HvAACT_T2_17A showed the highest relative root length, but all transgenic lines were significantly different from their respective nulls for relative second-longest root length (**Figure 3.9a**).

Total root length in Dayton was similar in acid and limed soils (**Figure 3.10a**). All other lines showed a decrease in total root length in the acid soil compared to the limed soil (**Figure 3.10a**). Significant differences in relative total root length were detected between homozygous lines HvAACT1_T2_17A and HvAACT1_T2_33A and their corresponding null lines but not between HvAACT1_T2_51 and its null sister line. (**Figure 3.10b**).

Representative photographs of some of the seedlings before processing are presented in **Figure 11**. The above experiments established that the null lines and Golden Promise were more sensitive to Al^{3+} than the homozygous transgenic lines and Dayton. The root diameters of these two groups were compared directly in the acid and limed soils. Distribution of root diameters in limed soil was similar for all lines (**Figures 3.12a and 3.12b**). In other words the total length of root in each thickness category was similar (equivalent to frequency) with peaks at about 0.15 and 0.44 mm. In acid soil root thicknesses increased for all transgenic and nulls lines in a similar way resulting in a single broader peak of 0.52 mm to 0.60 mm diameter (**Figure 3.12c** and **3.12d**). Even the Dayton plants were similar to the other lines in this experiment.

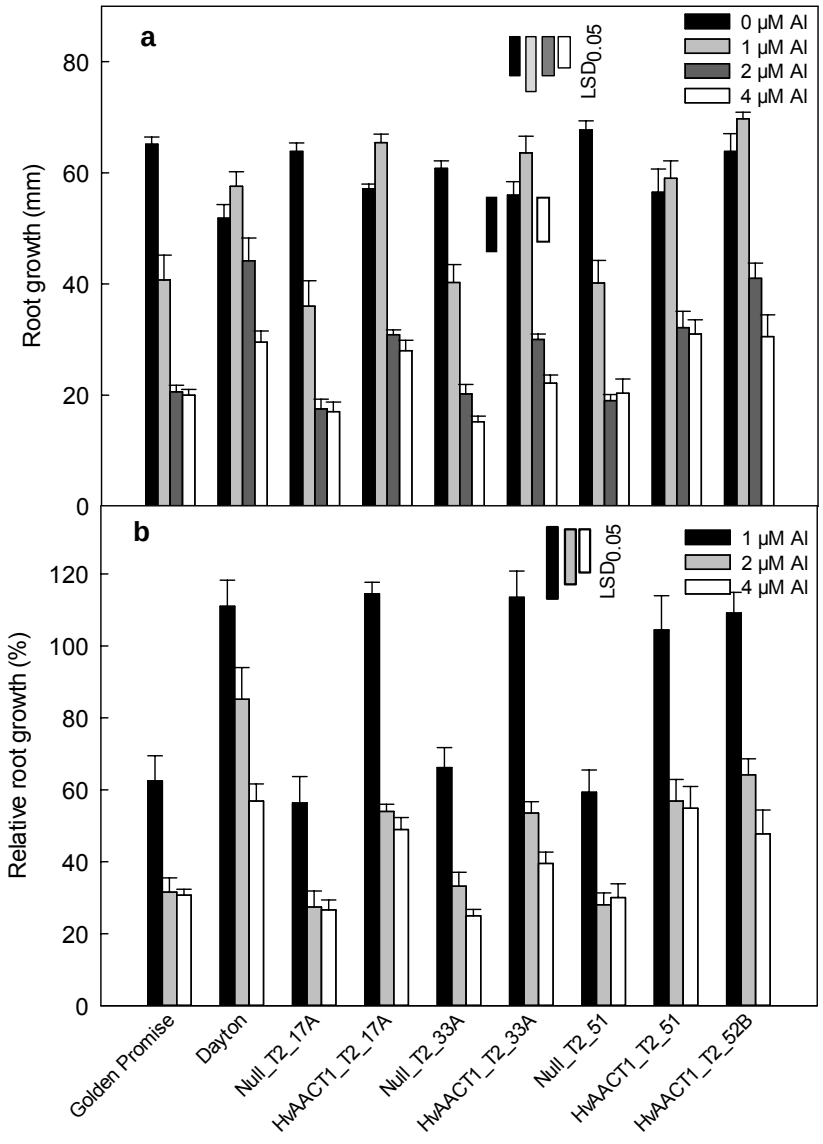


Figure 3.5 Al^{3+} resistance of the transgenic T2 homozygous lines and control lines in hydroponic culture. a. Root growth was measured before placing seed in hydroponics and after 4 days in nutrient solution containing $AlCl_3$. Data show the mean of root growth and standard error (n=7). b. Relative root growth in the same lines estimated after 4 days growth in hydroponic culture containing $AlCl_3$. Data show the mean of relative root growth and standard error (n=7). The bars represent the $LSD_{0.05}$.

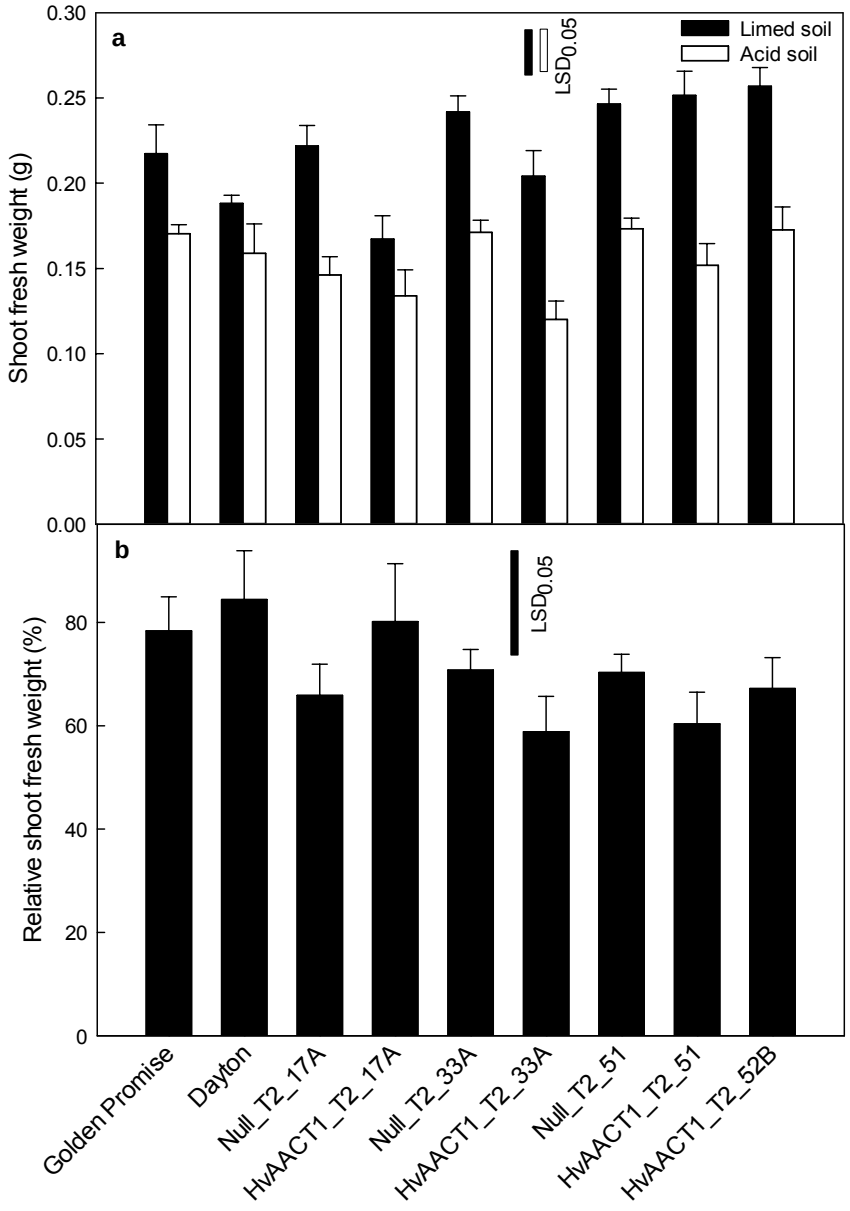


Figure 3.6 Shoot fresh weight of the transgenic T2 homozygous lines and control lines grown in soil. a. Shoot fresh weight of plants grown in acid soil and limed soil for 6 days. Data show the mean of shoot fresh weight (g) and standard error (n=6). b. The relative shoot fresh weight. Data show the mean of relative shoot fresh weight and standard error (n=6). The bars represent the $LSD_{0.05}$.

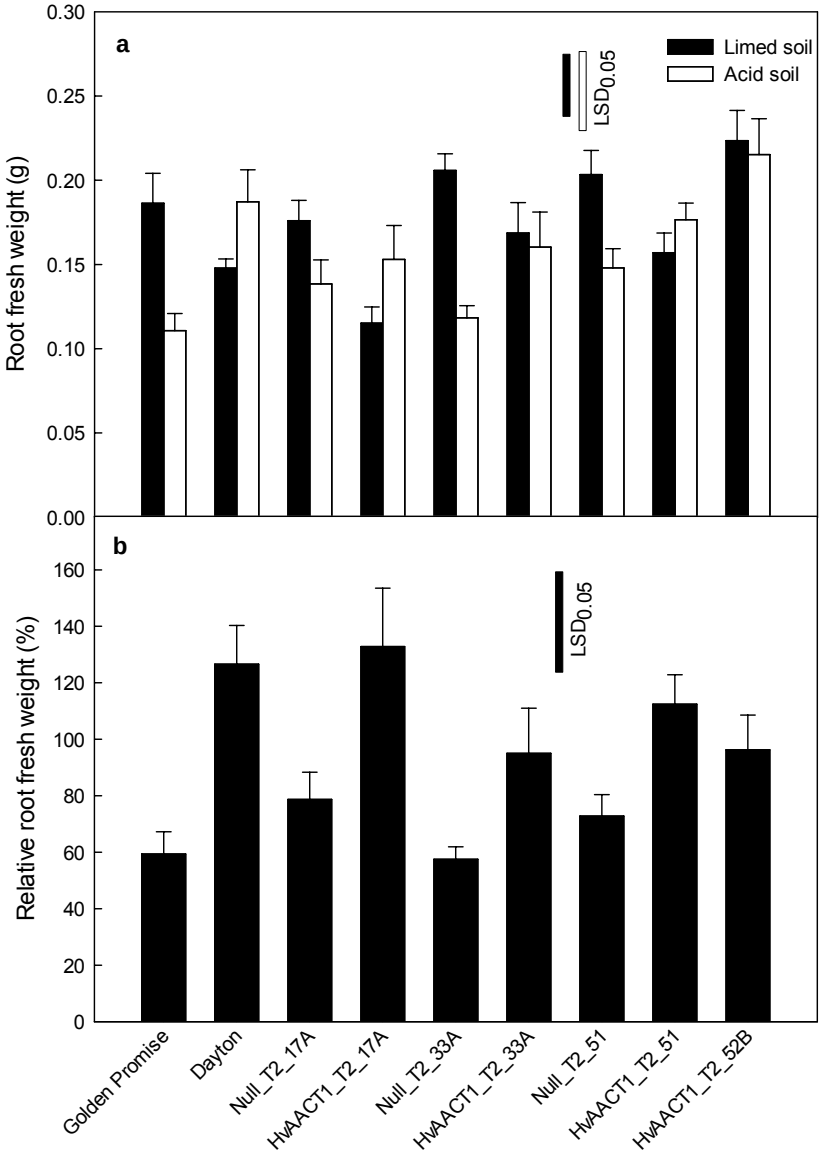


Figure 3.7 Root fresh weight of the transgenic T2 homozygous lines and control lines grown in soil. a. Root fresh weight of plants grown in acid soil and limed soil for 6 days. Data show the mean of root fresh weight (g) and standard error (n=6). b. The relative root fresh weight. Data show the mean of relative root fresh weight and standard error (n=6). The bars represent the $LSD_{0.05}$.

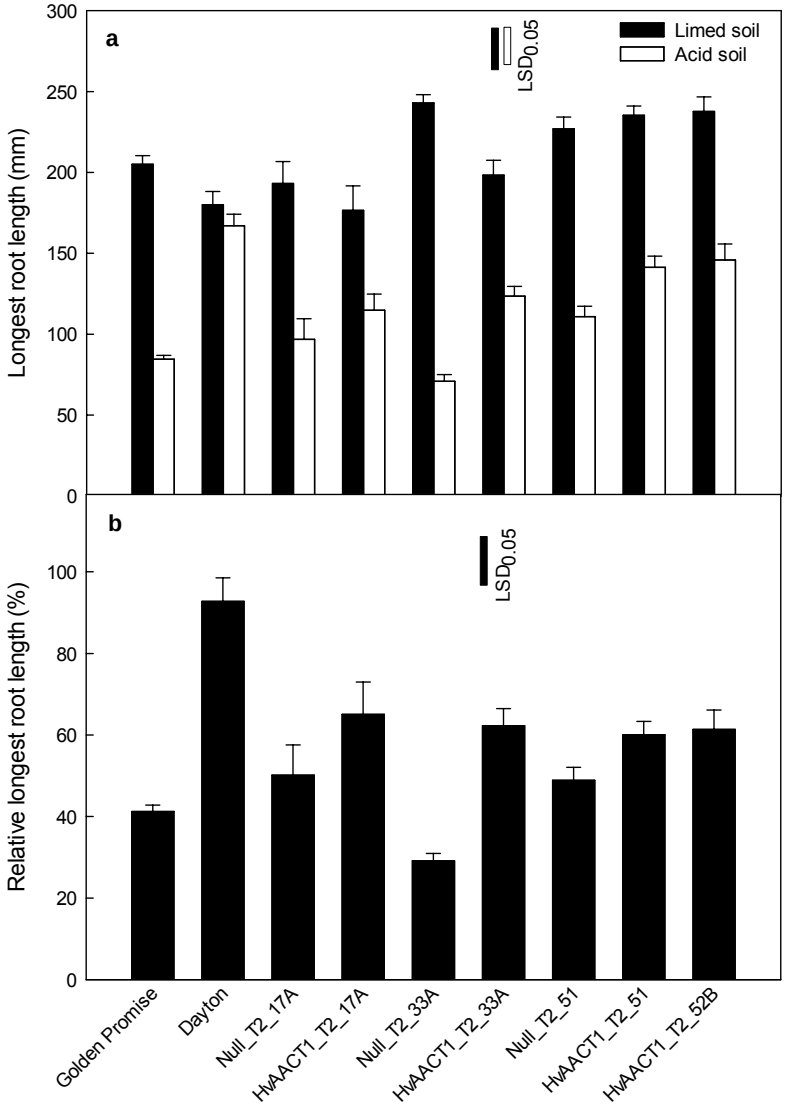


Figure 3.8 Longest root lengths in the transgenic T2 homozygous lines and control lines grown in soil. a. The longest root length from plants grown in acid soil and limed soil for 6 days. Data show the mean of the longest root length (mm) and standard error (n=6). b. Al^{3+} resistance was estimated in the same lines by comparing longest roots in the acid and limed soils. Data show the mean of relative longest root growth and standard error (n=6). The bars represent the $LSD_{0.05}$.

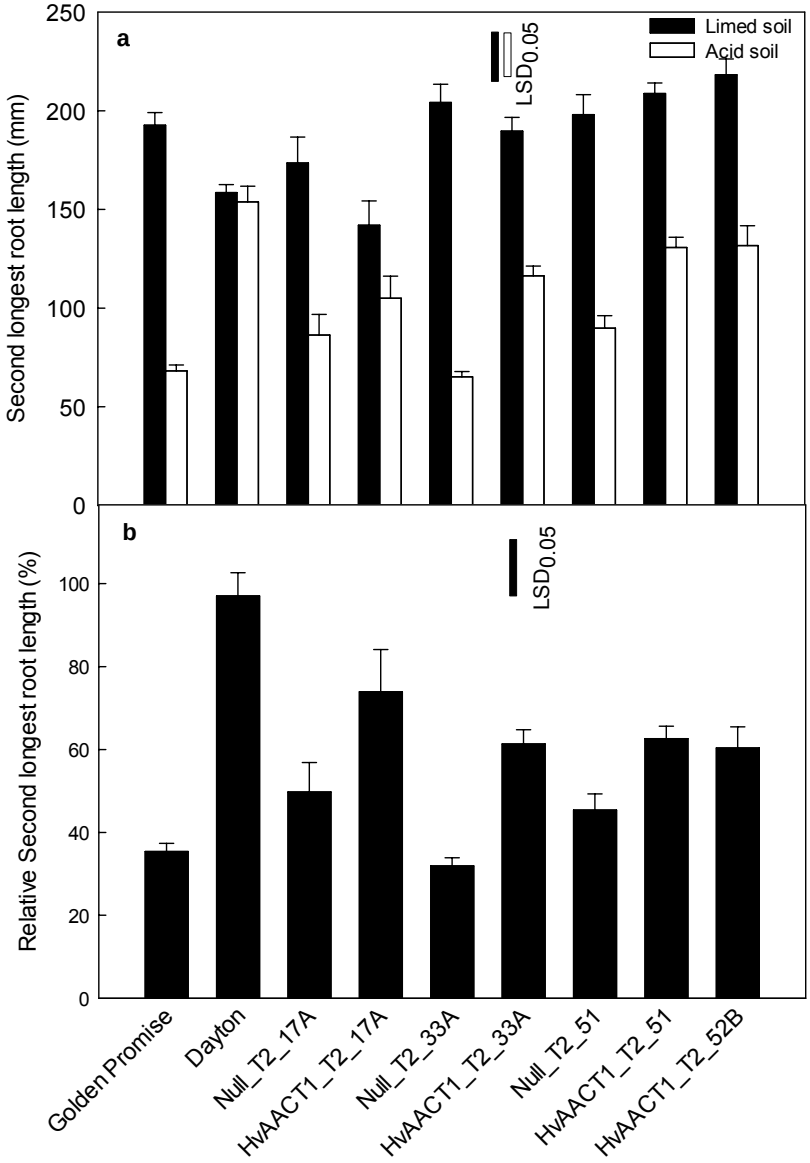


Figure 3.9 Length of the second-longest roots in the transgenic T2 homozygous lines and control lines grown in soil. a. The second longest root length from plants grown in acid soil and limed soil for 6 days. Data show the mean of the second-longest root length (mm) and standard error (n=6). b. Al^{3+} resistance was estimated in the same lines by comparing the second-longest root in acid and limed soils. Data show the mean of the relative second-longest root growth and standard error (n=6). The bars represent the $LSD_{0.05}$.

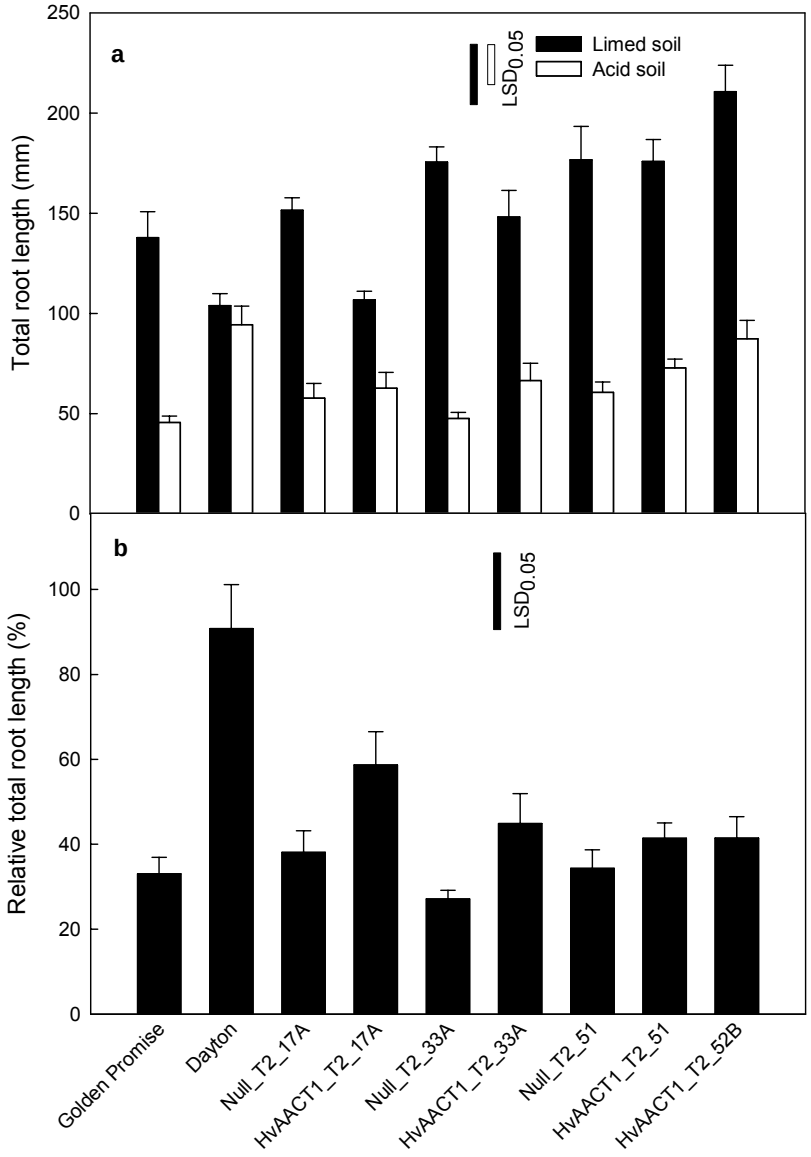


Figure 3.10 Total root length in the transgenic T2 homozygous lines and control lines grown in soil. a. Total root length after 6 days estimated from washed roots with the WinRHIZO scanner. The data show the mean of total root length in acid soil and limed soil and standard error (n=6). b.The relative total root growth in acid soil compared with limed soil. The data show the mean of relative total root growth and standard error (n=6). The bars represent the $LSD_{0.05}$.

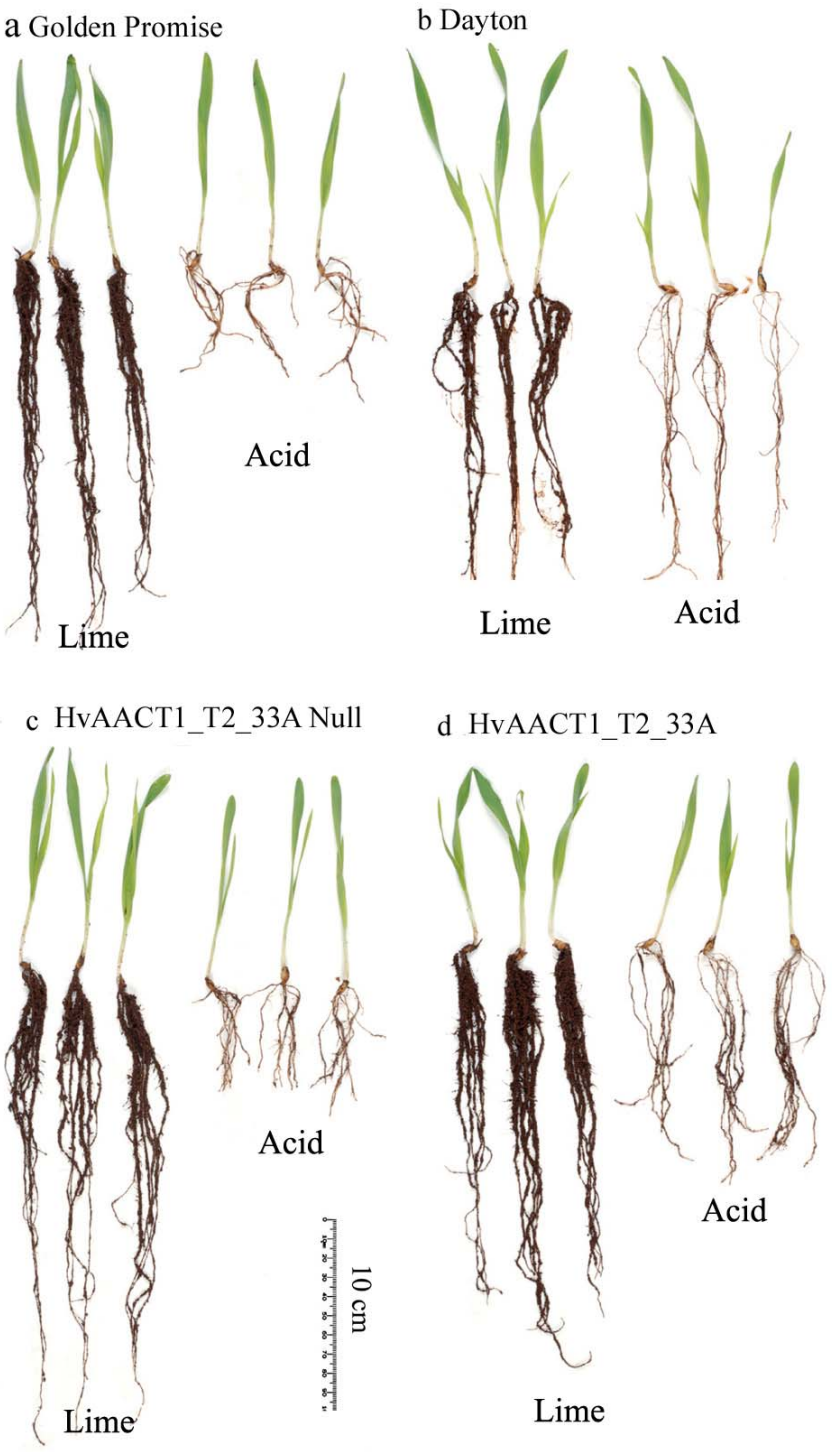


Figure 3.11 Examples of the transgenic and control plants taken from the soil experiments prior to processing. a. Golden Promise grown in limed soil (left) and acid soil (right); b. Dayton grown in limed soil (left) and acid soil (right); c. Null line T2_33A in limed soil (left) and acid soil (right); c. Homozygous line HvAACT1_T2_33A grown in limed soil (left) and acid soil (right).

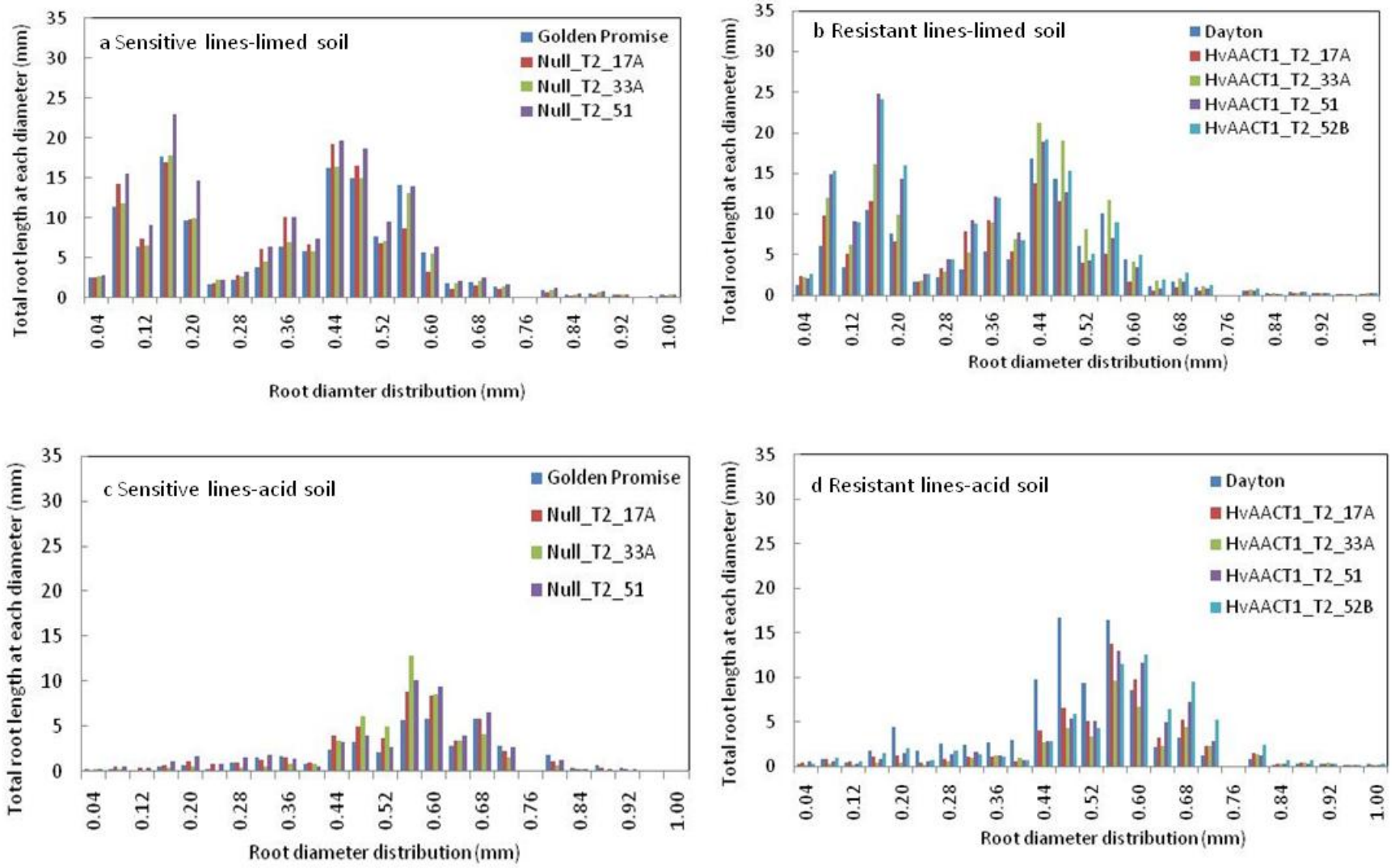

a Sensitive lines-limed soil
Golden Promise
Null_T2_17A
Null_T2_33A
Null_T2_51
Total root length at each diameter (mm)
Root diamter distribution (mm)
b Resistant lines-limed soil
Dayton
HvAACT1_T2_17A
HvAACT1_T2_33A
HvAACT1_T2_51
HvAACT1_T2_52B
Total root length at each diameter (mm)
Root diameter distribution (mm)
c Sensitive lines-acid soil
Golden Promise
Null_T2_17A
Null_T2_33A
Null_T2_51
Total root length at each diameter (mm)
Root diameter distribution (mm)
d Resistant lines-acid soil
Dayton
HvAACT1_T2_17A
HvAACT1_T2_33A
HvAACT1_T2_51
HvAACT1_T2_52B
Total root length at each diameter (mm)
Root diameter distribution (mm)

Figure 3.12

Figure 3.12 Distribution of root diameters from plants grown in soil. Previous experiments showed that the T2 homozygous lines and Dayton were more Al^{3+} resistant than the null lines and the parent cultivar Golden Promise. a. Frequency distribution of the root diameters of Al^{3+}-sensitive plants grown in limed soil. b. Root diameter distribution of Al^{3+}-resistant plants grown in limed soil. c. Root diameter distribution of Al^{3+}-sensitive plants grown in acid soil. d. Root diameter distribution of Al^{3+}-resistant plants grown in acid soil.

3.4 Discussion

The difference in Al^{3+} resistance of untransformed barley is determined by the expression level of *HvAACT1*. This gene encodes a transport protein which facilitates citrate efflux from the roots. The citrate is thought to bind with the toxic Al^{3+} to prevent it from damaging the sensitive roots apices (Furukawa et al., 2007). The barley cultivar used in this study (Golden Promise) is very susceptible to Al^{3+} toxicity. It shows little or no expression of *HvAACT1* in the root apices and very little Al^{3+}-activated efflux of citrate compared to the levels found in Al^{3+}-resistant barley. In this experiment, we found that transgenic barley lines over-expressing the *HvAACT1* gene had a high Al^{3+}-activated citrate efflux from root apices and greater Al^{3+}-resistance than wild-type in hydroponic solutions and in acid soil. No malate efflux was observed in these transgenic lines consistent with HvAACT1 facilitating citrate efflux but not malate efflux. These data support previous work that *HvAACT1* is a major citrate transporter gene for Al^{3+} resistance in barley.

The level of HvAACT1 expression and citrate efflux were not tightly correlated in the transgenic lines. *HvAACT1* expression among the putative homozygous T2 lines varied approximately eight-fold (**Figure 3.4**), yet citrate efflux was similar for all lines (**Figure 3.1**). Expression of the endogenous *HvAACT1* gene in Dayton was from 0.3 to 3.0 fold of that found in the transgenic lines yet citrate efflux from Dayton was approximately two-fold larger than all transgenic lines.

Interestingly, Furukawa et al. (2007) reported that there was a positive relationship between *HvAACT1* expression level and citrate efflux among barley genotypes. There are several reasons for the apparent discrepancy between this finding and the results presented here. Firstly, perhaps the number of transgenic lines analyzed here was insufficient to detect a statistical correlation between expression level and citrate efflux, if one does exist. Alternatively, the constitutive promoter in the

transgenic plants might obscure any correlation that might be present because the pattern of expression in roots of the transgenic plants differs from the pattern occurring naturally. In other words the correlation might only occur between citrate efflux and the expression in a specific cell or tissue type. It is also possible that other factors such as the supply of citrate or genetic background of barley influences the citrate efflux.

The transgenic lines performed better than the control lines in most Al^{3+} treatments in hydroponic solutions. When 1 μM Al^{3+} was included in the solution, root growth in the T2 homozygous lines was greater than in control solution without Al^{3+}. This stimulation of growth in acid condition by low levels of Al^{3+} has been observed previously and interpreted as Al^{3+} alleviating H^+ toxicity (Kinraide *et al.*, 1992).

When the transgenic plants were evaluated in acid soil, the data indicated that the fresh shoot weight was not a sensitive parameter for evaluating Al^{3+} resistance. The shoot is not directly exposed to Al^{3+} toxicity and any effects of Al^{3+} on the growth likely result from indirect effects on root growth such as their role in the uptake of water and nutrients. In short term experiments (6 d), there were no clear differences in shoots between Al^{3+} resistant lines and sensitive lines, but differences may become apparent with a longer growth period (Zhao et al., 2003). The relative longest root length, second longest root length and total root length showed similar trends among the lines with significant differences detected between the resistant lines (transgenic lines over-expressing *HvAACT1* and Dayton) and the sensitive lines (null lines and Golden Promise).

In limed soil the roots of all plants had a similar distribution of thicknesses with two peaks around 0.15 and 0.44 mm. In acid soil root thicknesses increased for the transgenic and nulls lines in a similar way (**Figure 3.12**). This is consistent with previous descriptions of Al^{3+} stress in acid soil where roots become shorter, thicker and, in some species, fewer in number (Foy, 1984, 1988). Even though the resistant lines maintained a greater total root length in the acid versus limed soils the final distribution of root diameters in the acid soil was similar in this experiment (**Figure 3.12**). Fewer, thicker roots will reduce the contact areas among roots and soils, and further decrease the uptake of nutrient and water in soil. This increase in root

thickness could explain the smaller differences in fresh root weights compared to total root length in the acid versus limed soils (**Figure 3.7**)

Differences in Al^{3+} resistance were detected among some of the controls. For instance the relative total root length of Golden Promise and three of the null lines was larger than Null_T2_33A (**Figure 3.10**) even though these lines should be genetically very similar. One possible reason for this might be that the seed used in the present experiments were harvested at different times or infected by fungi and therefore their germination rate and growth vigour may have differed. This demonstrates the importance of including transgenic controls (null segregant) in these types of experiments and, where possible, comparing seed derived from plants grown under the same conditions.

These results show that over-expression of *HvAACT1* can increase the Al^{3+} resistance of barley by increasing citrate efflux from root apices. The segregation analysis of T2_51 lines (**Table 3.1**) indicates that there is a single insert in this line, which shows low expression level of *HvAACT1* and citrate efflux (**Figures 3.1c** and **3.4**).

CHAPTER 4 Engineering aluminium resistance in barley with *SbMATE*

4.1 Introduction

The inheritance of Al^{3+} resistance in sorghum has been investigated in several studies. One of these investigated the F_2 progeny and $F_{2:3}$ families from crosses between parental lines BR007 (Al^{3+}-sensitive) and SC283 (Al^{3+}-resistant) and found that Al^{3+} resistance was controlled by a single locus. The QTL related to Al^{3+} resistance was mapped near the end of chromosome 3. An AFLP marker (*AFS37-1*) was found to be linked to Al^{3+} resistance. A molecular marker-based evaluation of Al^{3+} resistance diversity in sorghum revealed that the major Al^{3+} resistance loci were the same in three resistant sorghum cultivars (Magalhaes et al., 2004). Using a map-based cloning strategy, Magalhaes et al. (2007) identified an Al^{3+} resistance gene named *SbMATE*. It encodes a member of the multidrug and toxic compound exudation (MATE) family of transporter proteins (NCBI:ABS89149.1 GI:154986642). Sequence analysis of the Al^{3+}-resistant (SC283) and Al^{3+}-sensitive (BR007) cultivars revealed that polymorphisms in the coding region were found only in an intron region. Furthermore, there is a 728-bp indel about 1.4 kb upstream of the predicted TATA box for *SbMATE*. This region harbored a *Tourist*-like miniature inverted repeat transposable element (MITE). Magalhaes et al. (2007) also found a general correlation between the size of the MITE insertion in the promoter region with the level of *SbMATE* expression and aluminium resistance, but the expression level may also depend on additional interactions between this region and other sequences (Magalhaes et al., 2007).

SbMATE is an Al^{3+}-activated citrate transporter. There is no citrate efflux from either Al^{3+}-resistant or Al^{3+}-sensitive plants in the absence of $AlCl_3$. *SbMATE* expression is the highest in the apical region of the roots of Al^{3+}-resistant plants regardless of Al^{3+} treatment. Magalhaes et al. (2007) also found that *SbMATE* expression increased about 45% over 5 to 6 days in response to Al^{3+} treatment. The basal constitutive expression of *SbMATE* does not seem to be involved in Al^{3+} resistance. However the increase in *SbMATE* expression associated with Al^{3+} treatment was correlated with citrate efflux and Al^{3+} resistance. The SbMATE protein was localized to the plasma membrane when SbMATE-GFP fusion

constructs were transiently expressed in onion epidermal cells. Furthermore, *SbMATE* complementary RNA (cRNA) expression in *Xenopus laevis* oocytes generated inward ion currents consistent with SbMATE mediating anion movement across the membrane (Magalhaes et al., 2007).

This chapter describes experiments aimed at establishing whether Al^{3+} resistance in barley can be enhanced by expression of *SbMATE*.

4.2 Materials and Methods

The *SbMATE* cDNA (kindly provided by Dr Jurandir V. Magalhães, Embrapa Maize and Sorghum, Brazil) was partially digested using *Bam*HI and a 1,809 bp fragment was cloned into the pWUbi vector. Once the orientation of the cloned fragment was verified, a 4.2 kb *Not*I fragment was removed from pWUbi and cloned into the binary vector pWBVec8 (Wang *et al.*, 1998). The resulting plasmid was transformed into *Agrobacterium* strain Agl1. The *Agrobacterium* was used to generate seven T0 barley plants (cv. Golden Promise) from the 100 immature embryos initially infected.

The seven primary transgenic plants (T0) were grown in the greenhouse with natural light. The resulting T1 progeny were analyzed for citrate efflux with Al^{3+} treatment. Selected lines were grown to produce T2 and T3 seeds for further measurements. The Al^{3+} resistance of these transgenic lines was evaluated in hydroponic solution and soils. Citrate and malate efflux were assayed from root apices treated with or without 50 μM Al^{3+}. Real-time qRT-PCR was analyzed as described in Chapter 2. Primer 2F and 2R (**Table 2.1**) were used to amplify *SbMATE*, while primers 7F and 7R (**Table 2.1**) were used to amplify the *actin* gene in barley. Details of the methods are outlined in Chapter 2.

4.3 Results

4.3.1 Generation of homozygous lines

Seven independent T0 transgenic plant (9A, 22, 39, 98, 100E, 119 and 133) were planted in soil to generate T1 seeds. The T1 lines collected from these plants were screened for citrate efflux from the excised root apices. All resulting T1 lines except SbMATE_T1_39 had significantly greater citrate efflux compared to Golden

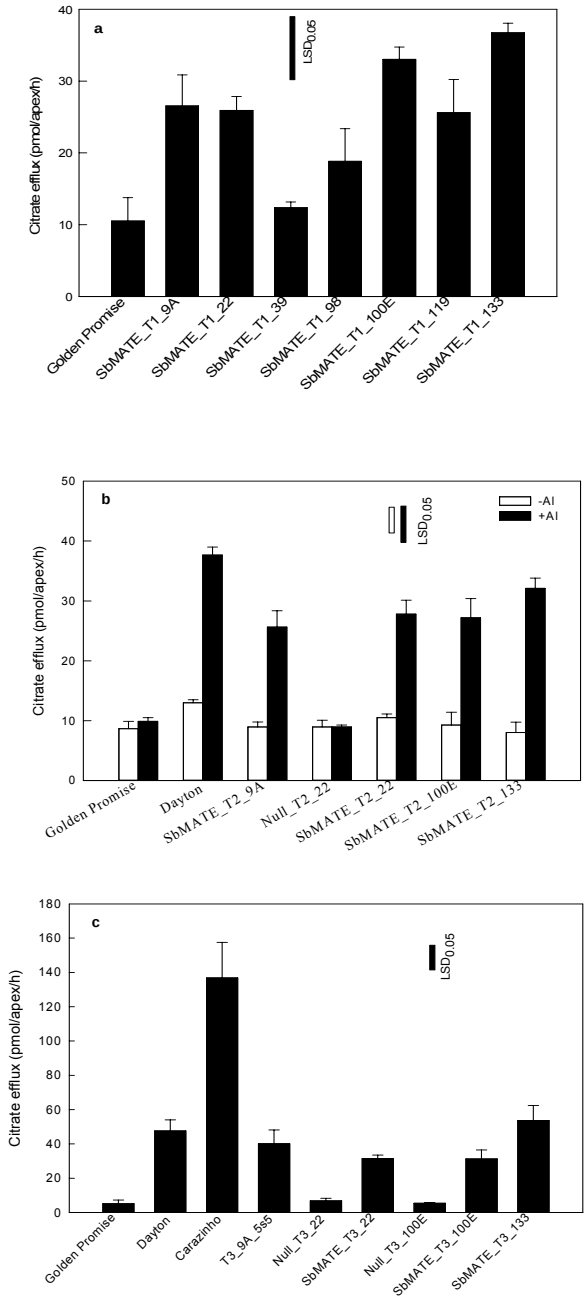


Figure 4.1 Citrate efflux from root apices of T1, T2 and T3 SbMATE transgenic lines. Also included in some experiments are the parental cv Golden Promise, Dayton (Al^{3+} resistant control) and Carazinho (Al^{3+} resistant wheat). a. Citrate efflux from excised root apices of selected T1 plants carrying *SbMATE* in mixture of homozygous and hemizygous plants with 50 µM Al^{3+} treatment. All of the lines except Golden Promise are *SbMATE* T1 lines. Data show the mean and standard error of citrate efflux (n=3). b. Citrate efflux in the T2 homozygous *SbMATE* transgenic lines with or without 50 µM Al^{3+} treatment. The suffix 'Null_T2' denotes a null line and 'SbMATE_T2' denotes homozygous T2 lines expressing *SbMATE*. Data show the mean and standard error of citrate efflux (n=3). c. Citrate efflux in the T3 homozygous *SbMATE* transgenic lines and null lines with 50 µM Al^{3+} treatment. Data show the mean and standard error of citrate efflux (n=4). The bars represent the $LSD_{0.05}$.

Promise (**Figure 4.1a**). Four T1 lines with the highest efflux (SbMATE_T1_9A, SbMATE_T1_22, SbMATE_T1_100E and SbMATE_T1_133) were chosen to generate T2 and T3 lines in a glasshouse. Null segregant plants from two of the transgenic lines were identified using the leaf assay for antibiotic resistance (**Chapter 2**). All of the lines were also screened for *SbMATE* insert using PCR, and the results were consistent with those from the leaf assay. Transgenic lines selected for further experimentation included T2_9A_5, T2_22_7, T2_22_8, T2_100E_1 and T2_133_9. These were renamed to SbMATE_T2_9A, SbMATE_T2_22 (T2_22_7), SbMATE_T2_100E and SbMATE_T2_133 for simplicity. Lines which were null for the transgene were T2_22-2 and T2_100E-4 and these were also renamed Null _T2_22 and Null _T2_100E for clarity. Lines with the same suffix (eg Null_T2_22 and SbMATE_T2_22) are sister lines (**Table 4.1**). The SbMATE_T2_22 and SbMATE_T2_100E might be homozygous for the transgene because approximately 25% of the T2 families are nulls (ie. all individuas in the family do not have the transgene) (**Table 4.1**) suggesting a 1:2:1 ratio of homozygous, hemizygous and null for the insert.

4.3.2 Organic anion efflux

The transgenic T2 lines were analyzed for citrate efflux and compared to the null line, the wild-type (Golden Promise) and the Al^{3+}-resistant cultivar Dayton. In the absence of Al^{3+}, low levels of citrate efflux (~10 pmol $apex^{-1}$ h^{-1}) were apparent from excised root apices of the T2 transgenic lines (**Figure 4.1b**). A proportion of this citrate was likely derived from the cut surfaces of the roots. In the presence of 50 μM Al^{3+} citrate efflux from Dayton and the transgenic lines expressing *SbMATE* increased approximately three-fold to about 30 pmol $apex^{-1}$ h^{-1}, Whereas the Al^{3+} sensitive parent cultivar Golden Promise and the line Null_T2_22 maintained low levels of citrate efflux in the presence of Al^{3+}.

Table 4.1 Screening T2 plants for homozygous lines of *SbMATE* transgenic barley

Lines	Transgenic	Non-transgenic	Total	Chosen
Line 9A				
T2_9A-1	14	5	19	
T2_9A-2	13	9	22	
T2_9A-3	3	9	12	
T2_9A-5	**19**	**0**	**19**	**Homozygous**
T2_9A-7	2	4	6	
Line 22				
T2_22-1	12	4	16	
Null_T2_22-2	**0**	**20**	**20**	**Null**
T2_22-3	13	5	18	
T2_22-4	17	4	21	
Null_T2_22-6	0	12	12	
T2_22-7	**19**	**0**	**19**	**Homozygous**
T2_22-8	15	0	15	
T2-22-10	3	2	5	
Line 100E				
T2_100E-1	**21**	**0**	**21**	**Homozygous**
T2_100E-2	14	5	19	
T2_100E-3	3	2	5	
Null_T2_100E-4	**0**	**22**	**22**	**Null**
Line 133				
T2_133-3	2	0	2	
T2_133-6	3	0	3	
T2_133-7	2	1	3	
T2_133-8	10	4	14	
T2_133-9	**26**	**0**	**26**	**Homozygous**

Citrate efflux from the root apices of T3 lines SbMATE_T3_133 and SbMATE_T3_9A was similar to Dayton (40 to 50 pmol $apex^{-1}$ h^{-1}), while efflux from SbMATE_T3_22 and SbMATE_T3_100E was slightly lower at 30 pmol $apex^{-1}$ h^{-1} (**Figure 4.1c**). Citrate efflux from Golden Promise and the null lines (Null_T3_22 and Null_T3_100E) was significantly lower (~5 pmol $apex^{-1}$ h^{-1}) (**Figure 4.1c**). Citrate efflux from the wheat genotype Carazinho is also shown. Carazinho is a Brazilian cultivar that shows constitutive efflux of citrate from its root apices (Ryan et al., 2009).

Malate efflux from all barley lines was low in the presence of 50 μM Al^{3+}. Malate efflux from the Al^{3+}-resistant wheat cultivar Carazinho, a positive control, was significantly higher (**Figures 4.2a and 4.2b**).

4.3.3 Transgene expression

The expression level of *SbMATE* from root tips was analyzed in the T3 transgenic lines. Lines SbMATE_T3_100E and SbMATE_T3_133 had three to five-fold greater expression level of *SbMATE* than SbMATE_T3_9A and SbMATE_T3_22 (**Figure 4.3).** *SbMATE* expression was not detected in any of the non-transgenic lines (Golden Promise, Dayton and Nulls) (**Figure 4.3**).

4.3.4 Relative root growth: hydroponic experiments

In the absence of Al^{3+}, root length among all lines varied from 50 mm to 80 mm after four days growth (**Figure 4.4a**). In the presence of 1 μM Al^{3+}, root growth of Golden Promise and null lines (Null_T3_22 and Null_T3_100E) was inhibited by about 40%. Increasing the Al^{3+} concentration to 2 μM and 4 μM Al^{3+} incrementally inhibited the root growth of all lines but more in Golden Promise and the null lines than in the transgenic lines expressing *SbMATE*. The root growth of these lines was about two to three-fold greater than the null lines (**Figure 4.4a**). Relative root growth (RRG) compares root growth at each Al^{3+} concentration with growth in the zero Al^{3+} control treatment. In three different Al^{3+} concentrations, the wild-type Golden Promise and the null lines had similar RRG. The RRG of the four transgenic lines expressing *SbMATE* was lower than that of Dayton, but remained two to three-fold greater than that of the wild-type and null plants and statistically different. RRG of the transgenic lines was about 120% in 1 μM Al^{3+} and about 70%

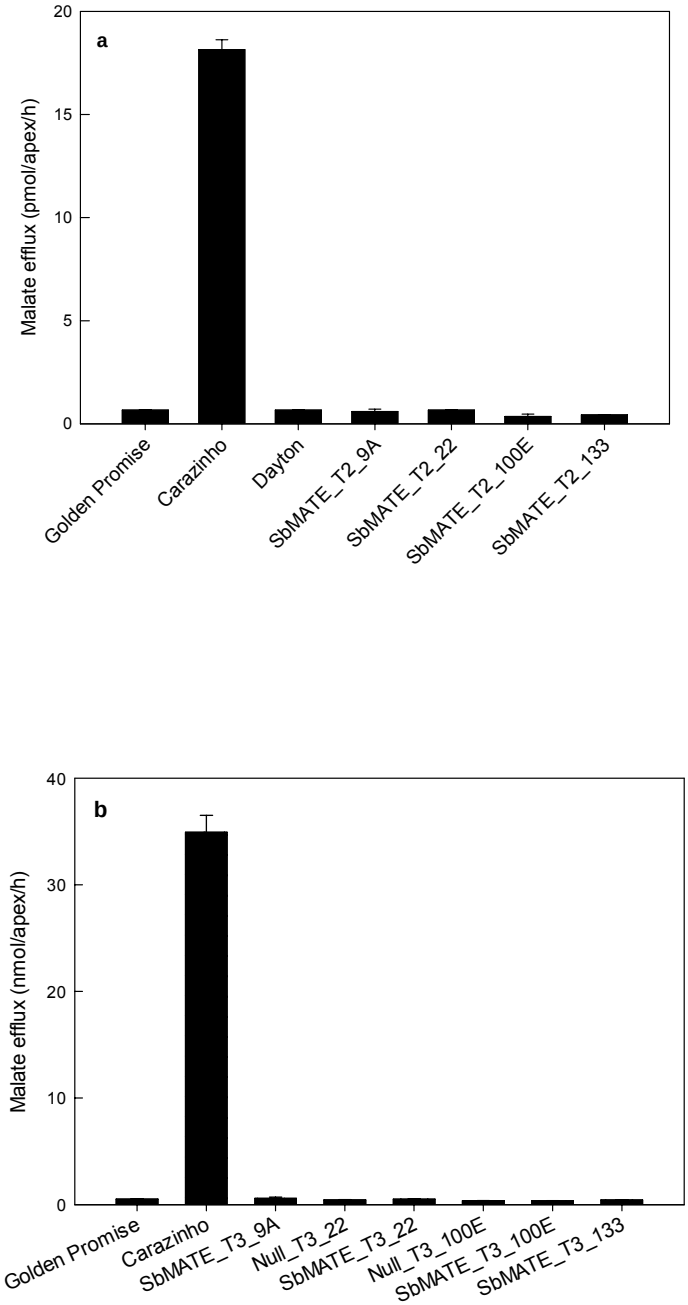


Figure 4.2 Malate efflux from root apices of T2 and T3 homozygous *SbMATE* transgenic lines in the presence of 50 µM Al^{3+}. Also included are the parental cv Golden Promise, Dayton (Al-resistant control); Carazinho, (Al-resistant wheat). a. Malate efflux of T2 homozygous *SbMATE* transgenic lines. 'SbMATE_T2_', *SbMATE* homozygous T2 lines. Data show the mean and standard error (n=3). b. Malate efflux from T3 homozygous *SbMATE* transgenic lines and null lines. Data show the mean and standard error (n=4).

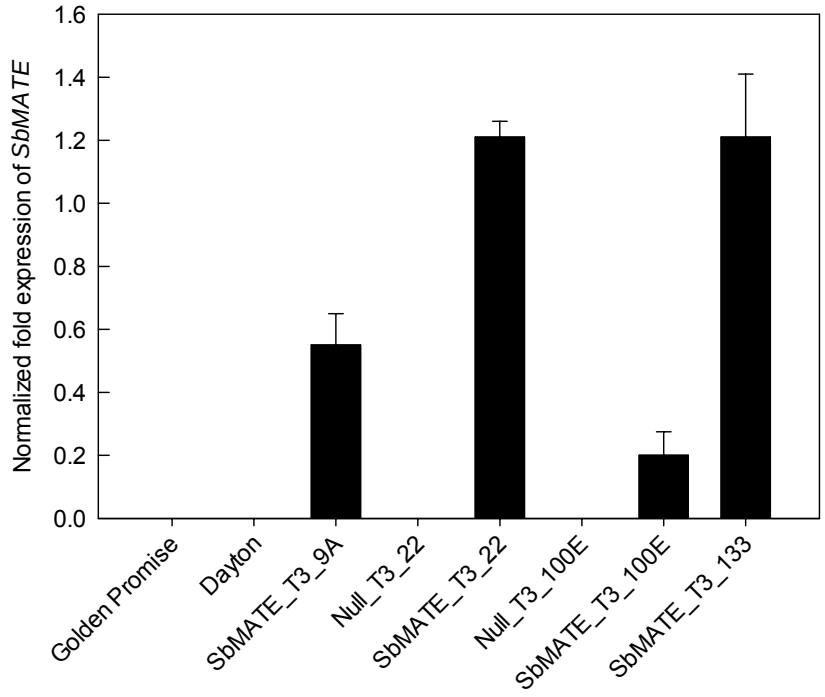


Figure 4.3 Normalized RNA expression level of *SbMATE* in T3 transgenic lines. Genotypes include the parental cv Golden Promise, Dayton (Al-resistant control); 'SbMATE_', *SbMATE* transgenics; Null_T3_22 and Null_T3_100E, nulls. Data show the mean and standard error of expression level compared to *actin* (n=4 biological replicates).

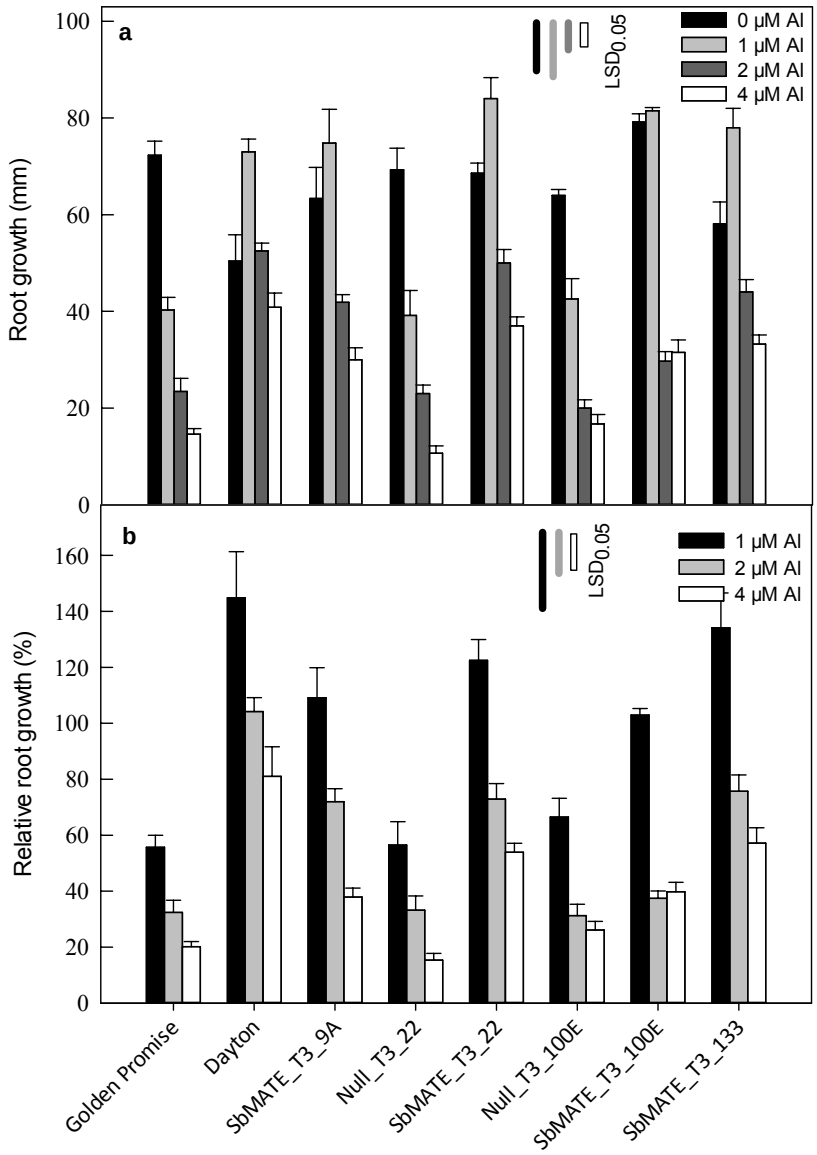


Figure 4.4 Al^{3+} resistance of T3 *SbMATE* transgenic lines and null lines in hydroponics. Also included are the parental cv Golden Promise, Dayton (Al-resistant control). a. Root growth after 4 days in nutrient solution containing 0 µM, 1 µM, 2 µM and 4 µM $AlCl_3$. Data show the mean of root growth and standard error (n=7). b. Relative root growth after 4 days in nutrient solution containing 1 µM, 2 µM and 4 µM $AlCl_3$. Data show the mean of relative root growth and standard error (n=7). The bars represent the $LSD_{0.05}$.

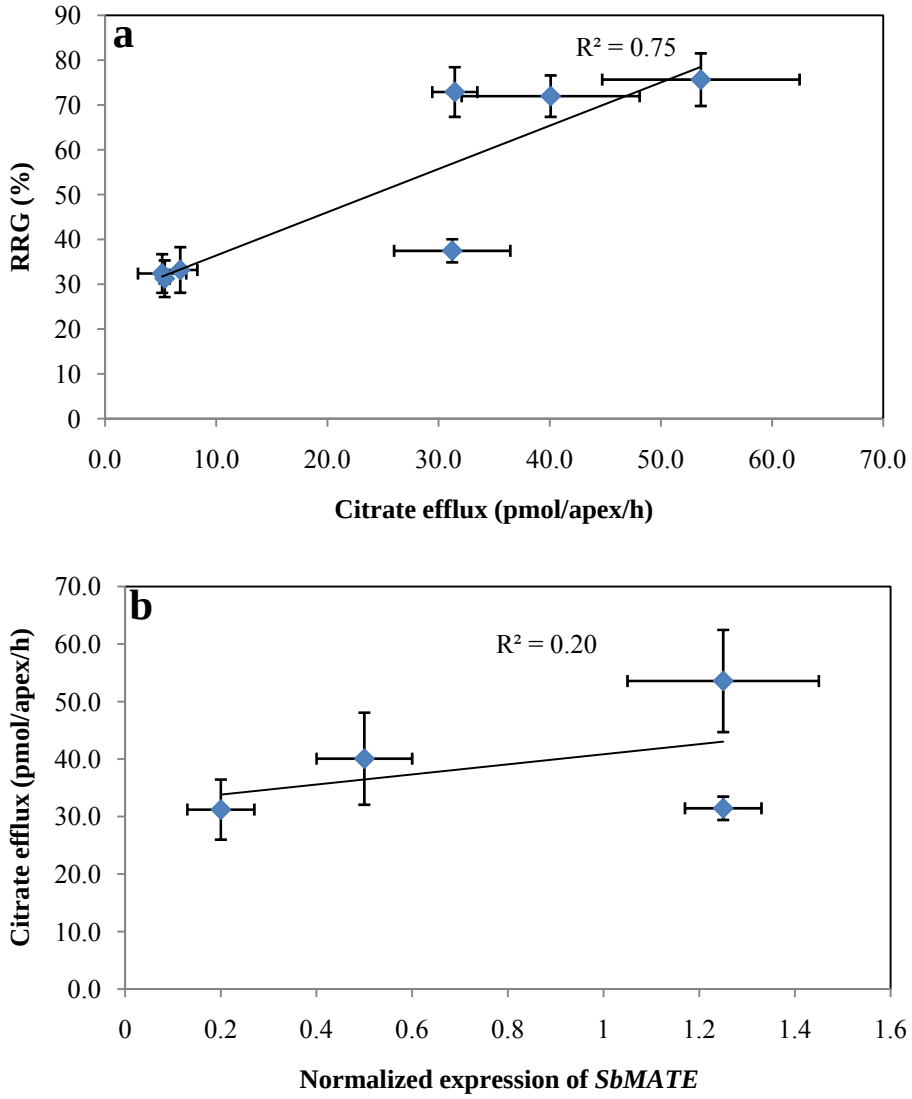


Figure 4.5 Relationships between *SbMATE* expression, citrate efflux and relative root growth (RRG) in T3 lines. a. Correlation between citrate efflux and RRG. RRG in 2 μM Al^{3+} hydroponic solution was used. b. Correlation between *SbMATE* expression and citrate efflux.

and 50% in 2 µM and 4 µM Al^{3+} respectively (**Figure 4.4b**). One of the transgenic lines, SbMATE_T3_100E, showed high Al^{3+} resistance in 1 µM Al^{3+} but not in 2 µM and 4 µM Al^{3+} condition.

A positive correlation exists between citrate efflux in the barley lines (four transgenic T3 lines, two null lines and Golden Promise) and their Al^{3+} resistance measured as RRG (**Figure 4.5a**) with a determination coefficient (r^2) of 0.75. The correlation was significant at the $P_{0.05}$ level. By contrast, the correlation between relative expression of *SbMATE* and citrate efflux in four transgenic T3 lines was lower with an r^2 of 0.20 (**Figure 4.5b**) and was not significant at $P_{0.05}$ level.

4.3.5 Relative root growth: soil experiments

Growth experiments were also conducted in an acid ferrosol soil with a pH of 4.3-4.5 (10 mM $CaCl_2$) (Chapter 2). Soil was either unamended or limed to raise the pH to 5.18 which reduced the toxicity of the soil caused by Al^{3+}. Plants were grown in pots in the glasshouse (Chapter 2). Measurements were made of fresh root and shoot weight (**Figures 4.7 and 4.8),** the longest root length and the second longest root length (**Figures 4.9 and 4.10**) and the total root length (**Figure 4.11**). The lines analyzed included the T3 transgenic lines, two null lines and the control cultivars Golden Promise and Dayton.

Figure 4.6 shows photographs of representative seedlings after removal from the soil. All of the lines show good rhizosheath in limed soil. While in acid soil, the parental line Golden Promise and null lines show loss of rhizosheath, which means smaller of root hairs. The transgenic line (**Figure 4.6b**) exhibits better.

No significant difference in absolute shoot fresh weight was detected between transgenic lines SbMATE_T3_22 and SbMATE_T3_100E and their respective null lines (Null_T3_22 and Null_T3_100E) in limed or acidic conditions (**Figure 4.7a**). Indeed the relative shoot weight in acid soil compared to the limed soil for transgenic and wild-type lines was similar (**Figure 4.7b**). Root fresh weight for all lines in limed soil was between 0.17 to 0.21 g (**Figure 4.8a**). In acid soil, the total fresh weight remained similar or increased slightly for all lines except for line Null_T3_100E which decreased in the acid soil by about 30% (**Figures 4.8a and 4.8b**).

Figure 4.6 Representative samples of *SbMATE* transgenic plants and non-transgenic plants grown in limed (left) and acid soils (right). In each photo left: Limed soil; right: acid soil. a) Null_T3_100E; b) SbMATE_T3_100E; c) Parental cv. Golden Promise; d) cv. Dayton.

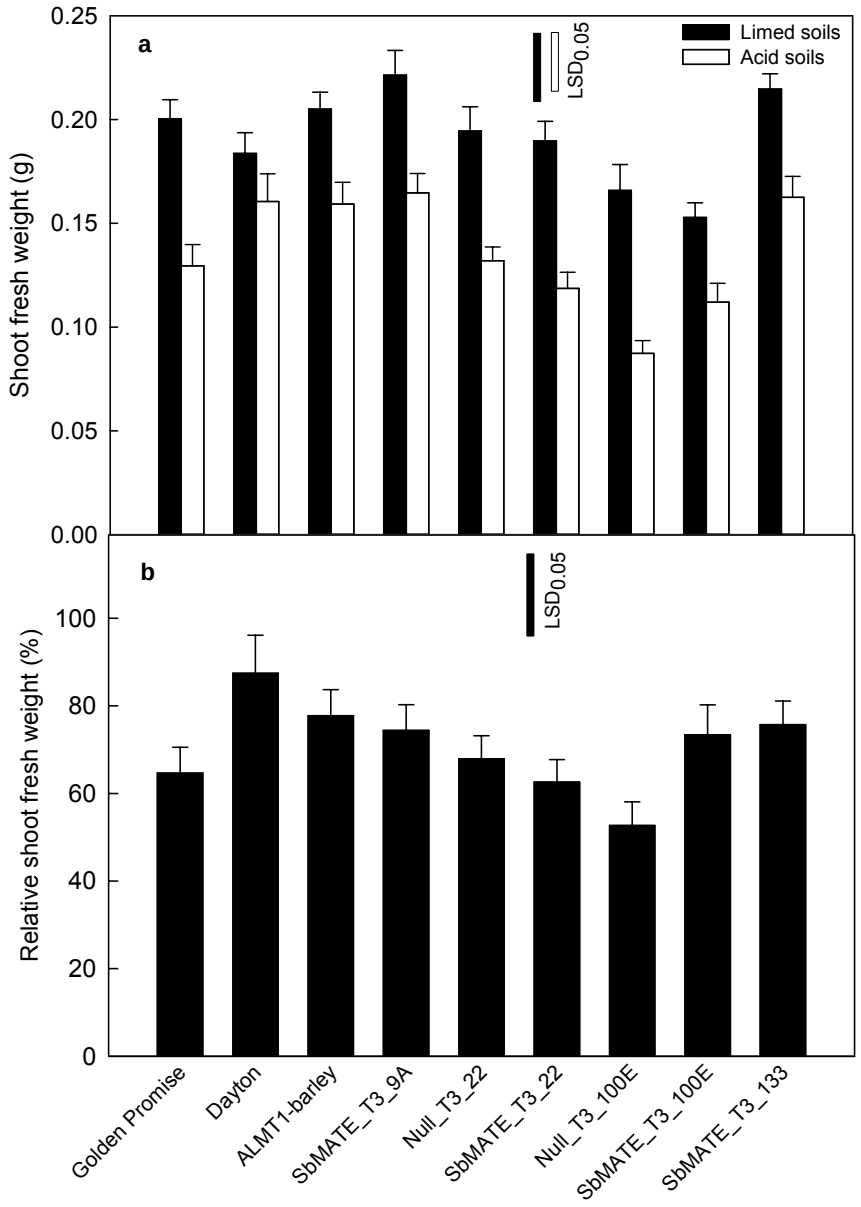


Figure 4.7 Shoot fresh weight grown in acid and limed soil. Genotypes include the parental cv Golden Promise, Dayton (Al-resistant control), transgenic barley plants expressing *TaALMT1* from wheat (ALMT1-barley), the transgenic T3 lines and null lines. a. Shoot fresh weight of T3 *SbMATE* transgenic lines in acid soil and lime soil. Data show the mean and standard error of shoot fresh weight (n=6). b. Relative shoot fresh weight of plants. White bars: Al^{3+} sensitive line, hatched bars: Al^{3+} resistant control lines and black bars: transgenic lines. Data show the mean and standard error of relative shoot fresh weight (n=6). The bars represent the $LSD_{0.05}$.

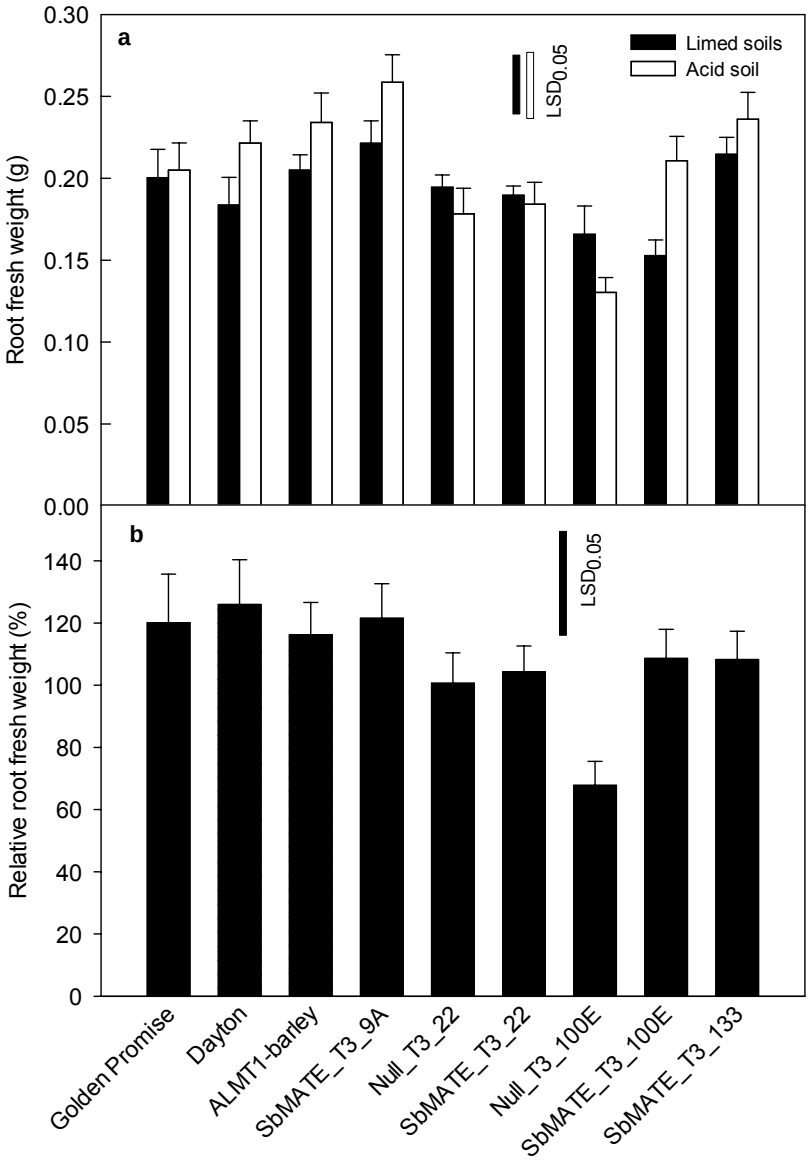


Figure 4.8 Effect of *SbMATE* expression on root fresh weight in acid and limed soil. Genotypes include the parental cv Golden Promise, Dayton (Al-resistant control), transgenic barley plants expressing *TaALMT1* from wheat (ALMT1-barley), the transgenic T3 lines and null lines. a. Root fresh weight of T3 *SbMATE* transgenic lines in acid soil and lime soil. Data show the mean and standard error of root fresh weight (n=6). b. Relative root fresh weight. Bars legends are the same to Figure 4.7. Data show the mean and standard error of relative root fresh weight (n=6). The bars represent the $LSD_{0.05}$.

In limed soil the average length of the longest and second longest root lengths were similar for all lines ranging from 160 to 200 mm (**Figures 4.9a and 4.10a**). In acid soil, three T3 transgenic lines (SbMATE_T3_9A, SbMATE_T3_100E and SbMATE_T3_133) and Dayton showed no reduction in these lengths while the fourth transgenic line showed a 17% reduction in the longest root but no change in the second longest root. This indicates that the root growth was relatively unaffected by acid soil in Dayton and the four transgenic lines expressing *SbMATE*. By contrast, the average longest root length in the two null lines (Null_T3_22 and Null_T3_100E) in acid soil was reduced by 40% and by 26% in Golden Promise compared to those in limed soil. Also included in this experiment is a transgenic barley line expressing the wheat Al^{3+}-resistance gene *TaALMT1* (ALMT1-barley). This transgenic line actually showed a ~14% increase in root length of the longest root in acid soil compared to the limed soil. Overall, similar responses were observed for the second longest roots except the differences between the transgenic and null lines in acid soil were larger. The relative changes in the longest roots and second longest roots lengths are summarized in **Figures 4.9b and 4.10b**.

Total root length in Dayton and transgenic barley expressing *TaALMT1* were the same in acid and limed soils (**Figure 4.11a**). All other lines showed a decrease in total root length in the acid soil compared to the lime (**Figure 4.11b**), with the greatest changes occurring for Golden Promise (45%), the two lines Null_T3_22 and Null_T3_100E (64% and 55% respectively). Three of the transgenic lines expressing *SbMATE* (SbMATE_T3_9A, SbMATE_T3_100E and SbMATE_T3_133) showed smaller decreases ranging from 18% to 30% while the fourth homozygous line (SbMATE_T3_ 22) showed an unexpectedly large decrease of 52% (**Figure 4.11b**).

Distribution of root diameters differed between transgenic lines and null in acid and limed soils. In limed soils, Al^{3+}-sensitive lines (Golden Promise and null lines) and Al^{3+}-resistant lines (Dayton, ALMT1 barley and transgenic lines) had similar distribution of root diameters. All lines had two peaks (**Figures 4.12a and 4.12b**), which meant that a large proportion of the roots had diameters near 0.16 mm or 0.46 mm. However, in acid soil, root diameters of the Al^{3+}-sensitive lines have one peak in the range of 0.56 mm to 0.60 mm. The Al^{3+}-resistant lines maintained two peaks at diameters of 0.20 mm and 0.56 mm (**Figures 4.12c and 4.12d**).

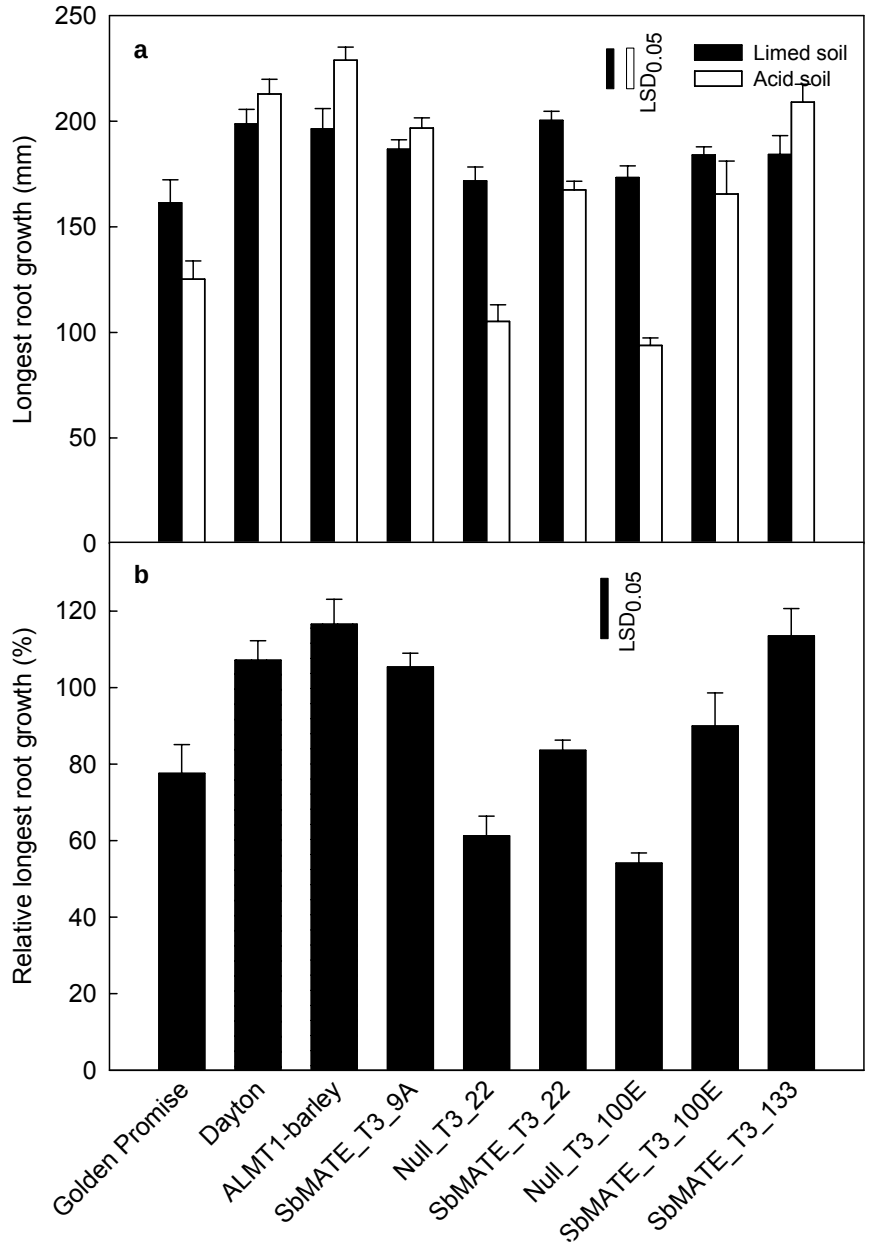


Figure 4.9 Effect of *SbMATE* expression on the length of the longest root. Genotypes include the parental cv Golden Promise, Dayton (Al-resistant control), transgenic barley plants expressing *TaALMT1* from wheat (ALMT1-barley), the transgenic T3 lines and null lines. a. The longest root growth of T3 *SbMATE* transgenic lines in acid soil and limed soil. Data show the mean and standard error of the longest root growth (n=6). b. Relative root growth of T3 *SbMATE* transgenic lines. Data show the mean and standard error of relative root growth (n=6). The bars represent the $LSD_{0.05}$.

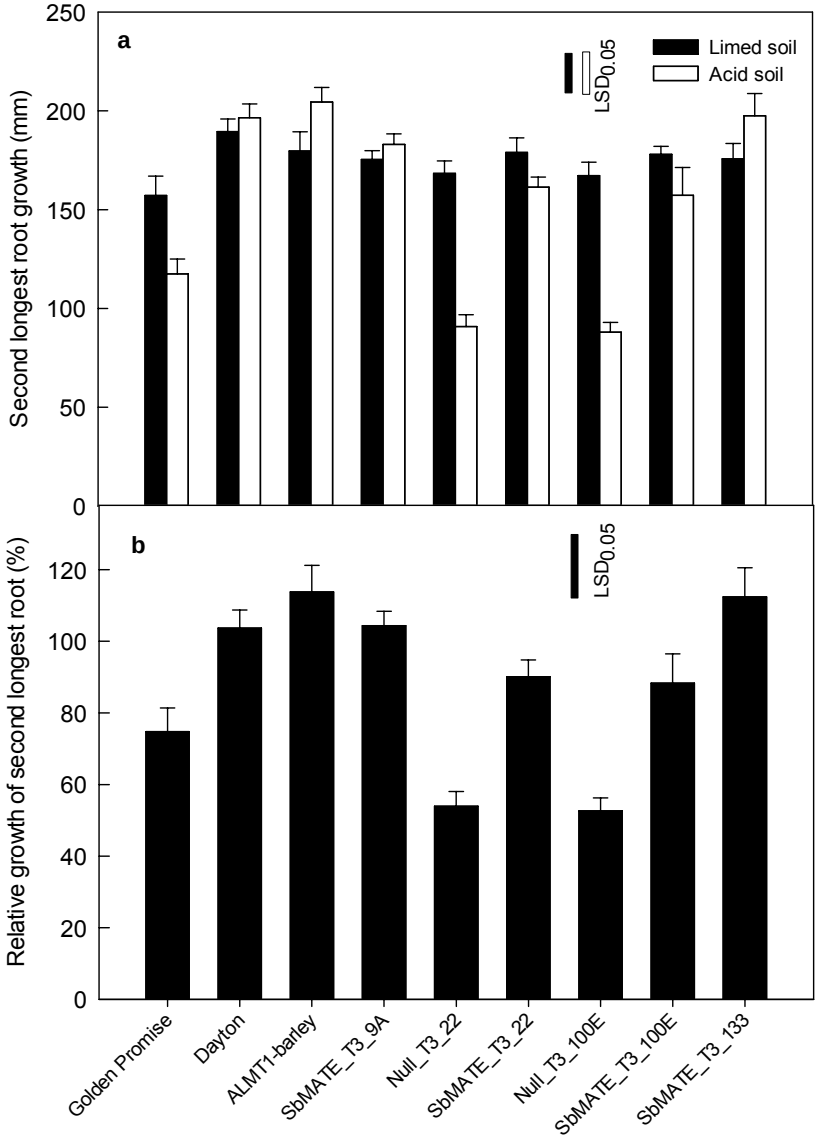


Figure 4.10 Effect of *SbMATE* expression on the length of the second longest root. Genotypes include the parental cv Golden Promise, Dayton (Al-resistant control), transgenic barley plants expressing *TaALMT1* from wheat (ALMT1-barley), the transgenic T3 lines and null lines. a. The second longest root length of T3 *SbMATE* transgenic lines in acid soil and lime soil. Data show the mean and standard error of the second longest root length (n=6). b. Relative second longest root growth of transgenic *SbMATE* T3 lines. Data show the mean and standard error of relative second longest root growth (n=6). The bars represent the $LSD_{0.05}$.

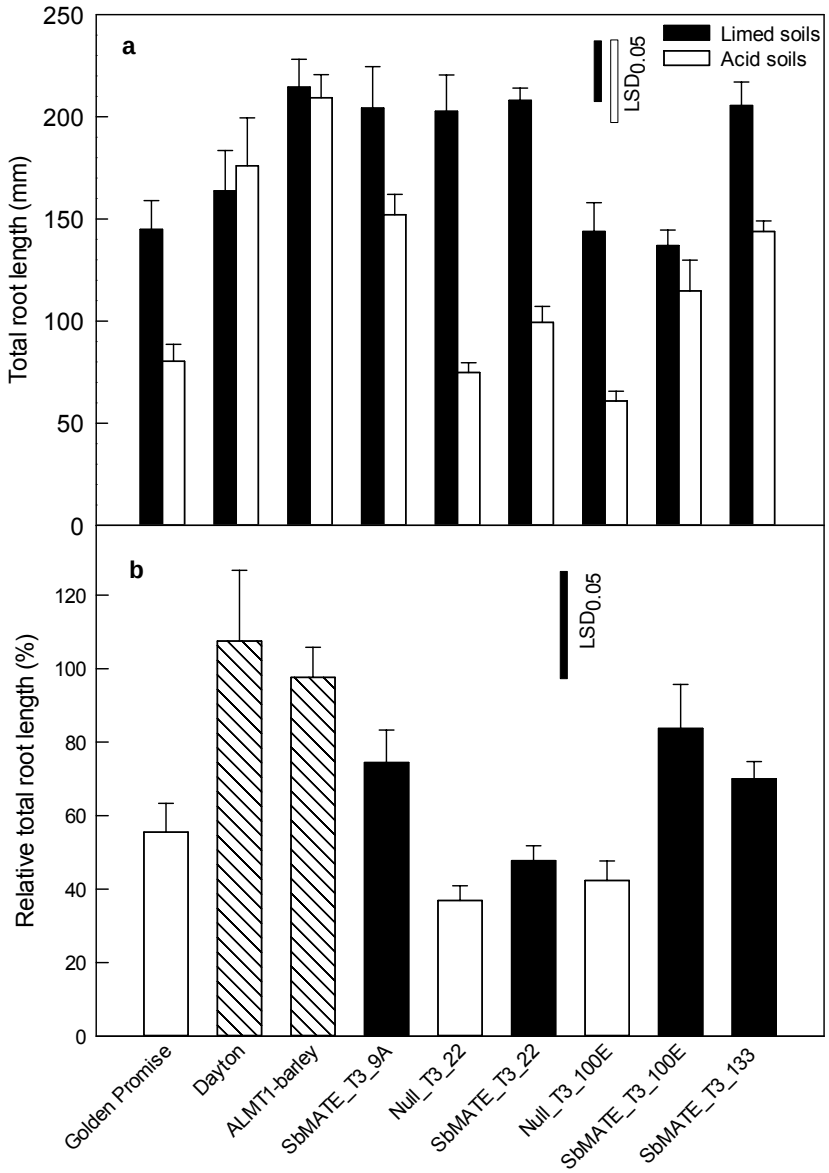


Figure 4.11 Effect of *SbMATE* expression on the total root length. Genotypes include the parental cv Golden Promise, Dayton (Al-resistant control), transgenic barley plants expressing *TaALMT1* from wheat (ALMT1-barley), the transgenic T3 lines and null lines. a. Total root length. The data show the total root length and standard error (n=6). b. Relative total root length. Bars legends are the same to Figure 4.7. The data show the mean and standard error of relative total root growth (n=6). The bars represent the $LSD_{0.05}$.

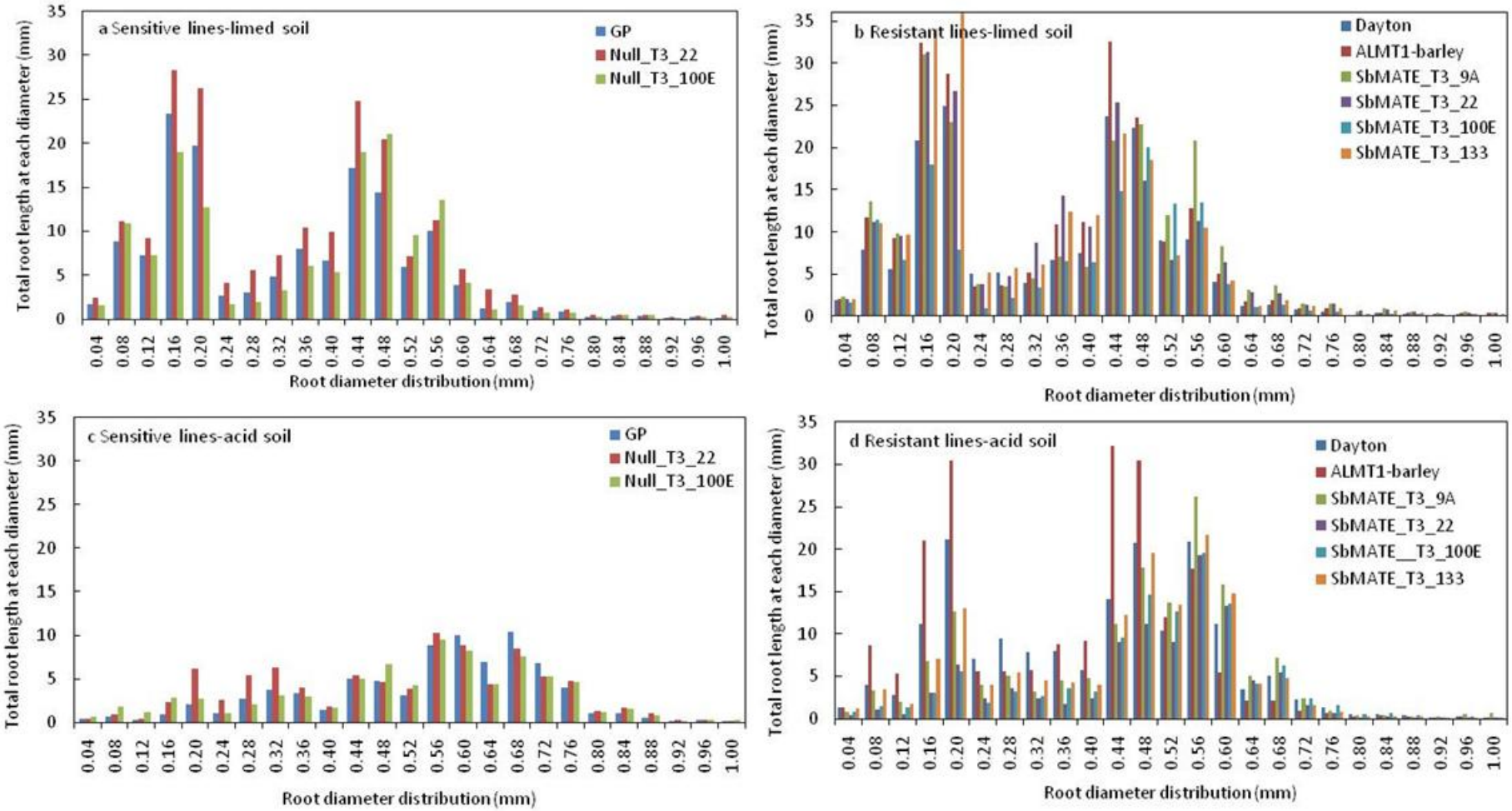

a Sensitive lines-limed soil
GP
Null_T3_22
Null_T3_100E
Total root length at each diameter (mm)
Root diameter distribution (mm)
b Resistant lines-limed soil
Dayton
ALMT1-barley
SbMATE_T3_9A
SbMATE_T3_22
SbMATE_T3_100E
SbMATE_T3_133
c Sensitive lines-acid soil
d Resistant lines-acid soil

Figure 4.12

Figure 4.12 Effect of *SbMATE* expression on the distribution of root diameters. Genotypes include the parental cv Golden Promise, Dayton (Al-resistant control), transgenic barley plants expressing *TaALMT1* from wheat (ALMT1-barley), the transgenic T3 lines and null lines. The Al-resistant lines were grouped together (ALMT1-barley, Dayton and transgenic T3 lines) and the Al-sensitive lines were grouped together (Golden Promise and Null lines). a. The distribution of root diameter of Al^{3+} sensitive plants grown in limed soil. b. The distribution of root diameter of Al^{3+} resistant plants grown in limed soil. c. The distribution of root diameter of Al^{3+} sensitive plants grown in acid soil. d. The distribution of root diameter of Al^{3+} resistant plants grown in acid soil.

4.4 Discussion

When the Al-sensitive barley cv. Golden Promise was transformed with *SbMATE*, it conferred an Al^{3+}-activated efflux of citrate and increased Al^{3+}-resistance. Among the measurements of resistance made the length of the longest and second longest roots and the total root length showed the greatest differences between the lines. In low Al^{3+} concentrations (eg. **Figure 4.4**) root growth of the T3 transgenic lines and Dayton was longer than in control solution without Al^{3+}. This stimulation of growth in acid conditions by low levels of Al^{3+} has been observed previously and interpreted as Al^{3+} alleviating H^+ toxicity (Kinraide et al., 1992). The null lines were even sensitive to low Al^{3+} concentrations and showed a decrease in root growth. These data provide evidence that *SbMATE* is capable of conferring Al^{3+}-resistance to whole plants and support a previous report proposing that *SbMATE* is a major gene for Al^{3+}-resistance in sorghum.

The phenotype conferred by *SbMATE* was maintained in T1, T2 and T3 generations in barley. Transgenic *Arabidopsis* plants expressing *SbMATE* also showed stable citrate efflux and Al^{3+} resistance in their T3 transgenic generations (Magalhaes et al., 2007). However expression of *SbMATE* in wheat was unstable past the T1 generation (L.V. Kochian, personal communication).

SbMATE expression increased citrate efflux from transgenic barley plants but the relationship between citrate efflux and relative expression level was not significant (**Figure 4.5b**). In other words, citrate efflux was not linearly related to transgene expression level (see comment earlier about this**)**. For example, the expression level of *SbMATE* in transgenic barley line SbMATE_T3_22 was about three-fold greater than that in SbMATE_T3_100E, while the citrate efflux of these two transgenic lines was similar (**Figure 4.1c**). This indicates that other factors other than

expression of this transport protein also limit the release of citrate from roots such as citrate supply. It could be instructive to compare the expression level of *SbMATE* in the transgenic lines with expression levels of *HvAACT1*, the endogenous gene controlling Al^{3+} resistance in barley. This is examined in detail in Chapter 6 (**Figure 6.2**).

Although the correlation between *SbMATE* expression and citrate was not good, the correlation between citrate efflux and Al^{3+} resistance was positive and significant. Lines SbMATE_T3_22 and SbMATE_T3_133 showed greater citrate efflux and greater Al^{3+} resistance than other transgenic lines. This result is similar to reports in sorghum (Magalhaes et al., 2007), wheat (Ryan et al., 1995b, Sasaki et al., 2004) and barley (Furukawa et al., 2007), showing correlations between organic anion efflux and Al^{3+} resistance in transgenic plants expressing other *MATE* and *ALMT* genes. Golden Promise and two null lines were included in Figure 4.5a while not in Figure 4.5b. Since there was no expression of *SbMATE* and citrate efflux in these Al^{3+}-sensitive lines, they were excluded from the Figure 4.5b which displayed the relationship between the expression level of *SbMATE* and the amount of citrate efflux. They were included in Figure 4.5a because these Al^{3+}-sensitive lines exhibited some citrate efflux, although whether it was linkage from root cut surface or not is unclear.

These experiments also show that the Al^{3+} resistance measured in hydroponics is greater in barley plants expressing the wheat *TaALMT1* gene than the sorghum *SbMATE* gene. For instance, *SbMATE*–expressing plants grown in hydroponics containing 4 μM Al^{3+}showed a RRG of about 60% to which was similar to the RRG for *TaALMT1*-expressing barley and wheat plants grown in 40 μM Al^{3+} under similar conditions (Delhaize et al., 2004, Pereira et al., 2010). This is examined further in Chapter 6. In the soil experiments conducted here, surprisingly, the performance of the *SbMATE* expressing barley lines except SbMATE_T3_22 was similar to the plants expressing *TaALMT1*. This inconsistency between hydroponic and soil performance has been reported by others in cereal screens. For instance, Moroni et al. (2010) found that although barley genotypes were more sensitive to Al^{3+} toxicity than wheat in hydroponics experiments, they did not differ nearly as much from wheat in acid soil trials. The Al^{3+}-resistant barley cultivar Dayton did

not differ significantly from the Al^{3+}-resistant wheat genotypes ET8 in acid soil despite showing large differences in hydroponic culture (Moroni et al., 2010).

When transgenic plants were evaluated in acid soil, the data indicated that the fresh shoot weight and fresh root weight were not sensitive parameters for evaluating Al^{3+} resistance. Even though the root apices are the most sensitive region to Al^{3+} stress and the root length of resistant lines was longer in limed soil than that in acid soil, the average root diameters in acid soil were much larger than those in limed soil (**Figures 4.12a and 4.12c**), resulting in fresh root weights in acid soil being similar to or larger than those in limed soil. In short term experiments (6d), there was no clear difference in shoots between Al^{3+} resistant lines and sensitive lines, but differences may become apparent with longer growth periods. The relative longest root length and the second longest root length showed similar trends among lines. Significant differences were also observed between resistant lines and sensitive lines in total root length. Measurements of total root length are too time-consuming for routine screening and the most efficient and effective way is to measure the longest root length in soil experiments. Interestingly, the relative root length of Golden Promise seedlings was higher than those of other null lines (Null_T3_22 and Null_T3_100E) even though these should be genetically very similar. Unlike Golden Promise, the nulls have been regenerated from callus in tissue culture which might affect plant metabolism and vigour. Furthermore, the Golden Promise seeds used in the present experiments were harvested at different times from the null lines and therefore their growth vigour might be different. This demonstrates the importance of including transgenic controls (null segregants) in these types of experiments and, where possible, comparing seed derived from plants grown under the same conditions.

The proportion of thin roots on Golden Promise and null lines decreased significantly in the acid soil compared to transgenic lines (**Figure 4.12c** and **4.12d**). Roots on Al^{3+}-sensitive plants become shorter, thickened and fewer in number in acid soil which will affect nutrient and water uptake. Similar observations have been made previously by Foy (1984).

CHAPTER 5 Al^{3+} resistance increased with expression of *Frd3* in barley

5.1 Introduction

In soils, iron exists primarily in the ferric (Fe^{3+}) form, and it is sparingly-soluble at neutral or basic pH. Even the very low concentrations of soluble iron in the soil solution exist as Fe(III) chelates. This poses a problem for plants to take up this essential nutrient and plants have evolved mechanisms that either increase iron solubility or improve the uptake of the chelated Fe(III). At the same time, the iron uptake should be regulated because excess iron can be toxic. There are two strategies used by vascular plants to acquire iron from the soil (Romheld, 1987). Eudicots and non-graminaceous monocotyledons rely on a set of iron deficiency responses termed strategy I that reduces Fe(III) to the more soluble Fe(II) form. Fe(II) is then taken up into roots by a specific transporter (Romheld, 1987). Grasses use a chelation-based mechanism termed strategy II, which involves the release of Fe(III)-specific chelators called phytosiderophores and the subsequent uptake of the Fe(III):phytosiderophore complex (Romheld, 1987).

Components of the strategy I response include the Fe(III) chelate reductase gene *FRO2* (*Ferric Reductase Oxidase2*) and the Fe(II) transporter *IRT1* gene (*Iron-regulated transporter1*) which were identified in *Arabidopsis* by complementation of yeast mutants defective in Fe(III) reductase activity or iron uptake (Eide *et al.*, 1996, Robinson *et al.*, 1999). The expression of both genes is upregulated by iron deficiency (Vert *et al.*, 2002).

Three *Arabidopsis* mutants have been identified which show enhanced Fe(III) reductase activities in their roots under iron-sufficient conditions (Delhaize, 1996, Rogers and Guerinot, 2002, Yi, 1995). This phenotype is controlled by recessive mutations in a member of the *MATE* gene family named *FRD3* (Rogers and Guerinot, 2002). The three mutants had single nucleotide changes in *FRD3*. *frd3-1* has a C to A transversion causing the substitution of Asp for Ala, while *frd3-2* has a deletion of a single G causing a frameshift, the addition of seven amino acids after the deletion and a premature stop codon. *frd3-3* has a G to A transition in the first nucleotide of the fifth intron, which results in the introns being retained and

addition of two novel amino acids followed by a premature stop codon. Interestingly, plants homozygous for any of the three mutant alleles had *FRD3* mRNA levels considerably greater than the wild-type. All three mutants had increased concentrations of iron in the shoots compared to wild type plants. Furthermore, *frd3* mutants exhibit constitutive expression of other iron-deficiency responses including the up-regulation of *IRT1* and *FRO2*. When the *Frd3* cDNA was fused to the green fluorescent protein (GFP) gene and expressed in *Arabidopsis* mutant *frd3-1* under the control of the *Frd3* promoter, it was able to complement the mutant phenotype. *Frd3* is expressed in the pericycle and cells internal to the pericycle and surrounding the vascular tissue (Green and Rogers, 2004). These phenotypes are consistent with defects either in the signaling pathways involved with detecting iron deficiency or in the translocation and localization of iron to the shoots. Although the iron content in shoot tissues was higher in *frd3* mutant plants, Green and Rogers et al. (2004) showed that the distribution of iron was altered in the mutant. Histochemical staining indicated that the *frd3* mutants accumulated high levels of ferric iron in the vascular cylinder of roots and shoots, where the *FRD3* gene is expressed. Green and Rogers (2004) suggested that the likely function of FRD3 was to load an iron chelator into the root xylem or other compounds necessary for the efficient re-absorbtion of iron out of the xylem or apoplastic space and into leaf cells. Subsequently, Durrett et al. (2007) showed that *frd3* plants contain significantly less citrate in xylem exudates than wild-type plants. Reciprocal grafting experiments further showed that *FRD3* function was root-specific. Heterologous expression of *FRD3* in *Xenopus laevis* oocytes generated citrate-dependent currents and expression in *Arabidopsis* plants resulted in a three-fold greater efflux of citrate from the roots (Durrett et al., 2007). This citrate efflux from the transgenic *Arabidopsis* plants increased the resistance of those plants to Al^{3+} toxicity. These results are consistent with FRD3 mediating citrate efflux into the root vasculature of wildtype plants, a process important for the translocation of iron to the leaves. This also supports previous suggestions that iron moves through the xylem as a ferric-citrate complex (Durrett et al., 2007). Therefore *FRD3* mediates citrate efflux from the xylem parenchyma to the xylem which is necessary for the efficient translocation of iron to the leaves.

Other members of the *MATE* gene family confer Al^{3+} resistance by facilitating citrate efflux from the roots of barley, sorghum, wheat, maize, rye, rice and bean (See Chapter 1). FRD3 is not involved with conferring Al^{3+} resistance in natural ecotypes of *Arabidopsis* analyzed to date and is unlikely to be exposed to Al^{3+} *in vivo* because it is expressed in the vasculature tissue and not at the root surface. Therefore, it is not expected that FRD3 would be activated by Al^{3+}. Transgenic plants over-expressing FRD3 are likely to release citrate constitutively as was reported for the transgenic *Arabidopsis* mentioned above (Durrett et al., 2007). The aim of this Chapter is to determine whether expression of the *Frd3* gene from *Arabidopsis* can increase the Al^{3+} resistance of transgenic barley.

5.2 Material and methods

The cDNA of *Frd3* was kindly provided by Elizabeth Rogers (University of Missouri, USA) and the Kazusa DNA Research Institute (Chiba, Japan; www.kazusa.or.jp). The cDNA was ligated into the pBluescript II SK plasmid at the *Eco*RI and *Xho*I sites (5' to 3'). This plasmid was sequentially digested with *Kpn*I and then *Bam*HI and the resulting 1.8 kb band containing *Frd3* was gel purified and ligated into pWUbi also digested with *Kpn*I and *Bam*HI. The resulting plasmid was transformed into *E.coli* and minipreps prepared from the antibiotic resistant colonies. The pWUbi :Frd3 plasmid was digested with *Not*I which released a 4.2 kb fragment containing the maize ubiquitin promoter and a maize ubiquitin intron upstream of the *Frd3* cDNA with the *tml* terminator. This fragment was cloned into the *Not*I site of the pVec8 binary vector and transformed into *E.coli* and then *Agrobacterium*. The *Agrobacterium* was used to transform barley cultivar Golden Promise (GP).

T1 seeds were grown to generate T2 and T3 lines homozygous for the insert. Al^{3+} resistance of these transgenic lines was evaluated in hydroponic solution with 0μM, 1 μM, 2 μM and 4 μM Al^{3+}. They were also identified in acid soil and limed soil. Citrate and malate assay from root apices was treated with or without 50 μM Al^{3+}. Real-time PCR were analyzed. Other details are described in Chapter 2.

Shoot and roots were collected from *Frd3* T3 lines and Golden Promise plants grown with a low and sufficient iron concentration. Between 3 to 10 plants per line

were grown in aerated hydroponic solution with either 5 μM or 100 μM $FeCl_3$ for ten days. Shoot and root tissues were collected and dried at 60^oC for 2 days for elemental analysis. Approximately 40 mg of the dried samples were extracted with di-ammonium ethylenediaminetetraacetic acid (EDTA) solution and the extractants used for mineral analysis using ICP-MS spectrometry (IRIS/APHRDUO Thermo Electron Corporation, Franklin, MA, USA).

5.3 Results

5.3.1 Generation of transgenic lines

The Al^{3+}-sensitive barley cultivar 'Golden Promise' was transformed with *Frd3* driven by the constitutive promoter from the ubiquitin gene. Eight T0 transgenic lines (38A, 40, 52A, 55, 58A, 42, 49, 83) were obtained from the 100 embryos treated with *Agrobacterium*.

5.3.2 Analysis of T1 lines

T1 seed were harvested from the T0 plants and analyzed for citrate efflux. All the transgenic lines except line 38A and 52A exhibited greater citrate efflux than the parental cultivar Golden Promise in the presence of 50 μM $AlCl_3$ (**Figure 5.1a**). Aluminium was included in these experiments although it was later demonstrated that citrate efflux occurs in the absence of Al^{3+} (see below). T1 families will segregate for the transgene and therefore individual plants will either be homozygous or hemizygous for the *Frd3* gene or null segregants. Segregation of T1 lines was confirmed by leaf assay method for hygromycin resistance (See Chapter 2). Lines T1_40 and T1_55 showed the highest citrate efflux. The ratio of resistant plants to sensitive plants was 9:3 for line T1_40 and 11:4 for line T1_55. These ratios are not different from a 3:1 segregation ratio (χ^2 tested at $P_{0.05}$ level)**,** which is consistent with a single gene insert. Twelve plants of each of the T1_40 and T1_55 lines (including one null plant) were grown to generate T2 seed.

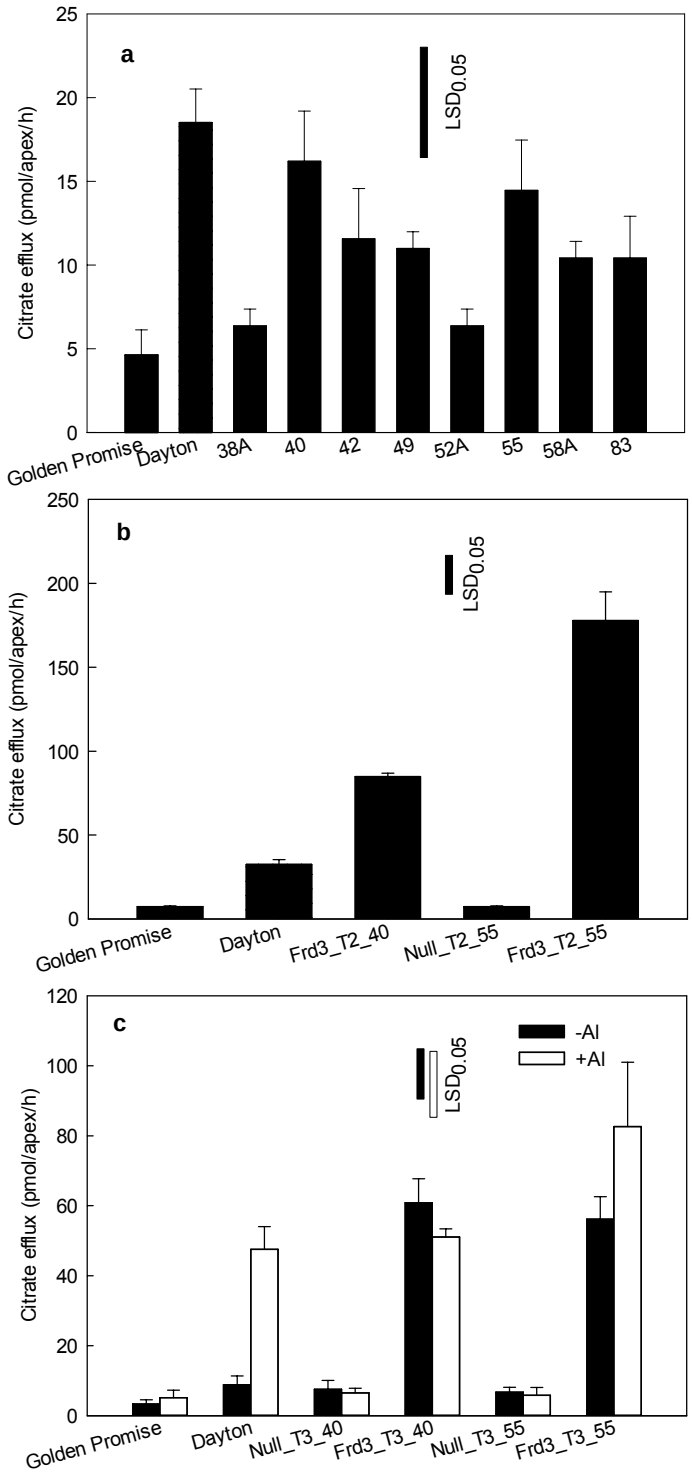


Figure 5.1 Citrate efflux from root apices of transgenic and control plants. a. Citrate efflux in the T1 *Frd3* transgenic lines. Citrate efflux was measured from excised root apices of selected plants expressing *Frd3* in the presence of 50 µM $AlCl_3$. Data show the means and standard error (n=3). b. Citrate efflux in the T2 homozygous *Frd3* transgenic lines. Citrate efflux was measured from 8 excised root apices with 50 µM $AlCl_3$ treatment. Data show the mean and standard errors (n=4). c. Citrate efflux in the T3 homozygous *Frd3* transgenic lines. Citrate efflux was measured from 8 excised root apices with or without 50 µM $AlCl_3$ treatment. Data show the means and standard errors (n=4). The bars represent the $LSD_{0.05}$.

5.3.3 Analysis of T2 plants

Potential homozygous lines among the T2 families were identified by scoring leaves from individual plants for resistance to hygromycin. Although seed for many lines were limited, the lines T2_40-3, T2_40-7, T2_40-9, T2_55-2, T2_55-3 and T2_55-7 were possible homozygous lines, while T2_40-11 and T2_55-1 were null lines (**Table 5.1**). The ratio of homozygous to heterozygous among the T2 families supports the suggestion that they contain a single copy of the transgene. The potential homozygous lines T2_40-7 and T2_55-3 were named to Frd3_T2_40 and Frd3_T2_55 for simplicity, while their sister lines without *Frd3* were named to Null_T2_40 and Null_T2_55.

Citrate efflux from lines Frd3_T2_40 and Frd3_T2_55 was greater than that from the parental cultivar Golden Promise and the null line Null_T2_55 and even greater than the Al^{3+}-resistant cultivar, Dayton (**Figure 5.1b**). Root growth of the T2 homozygous and control lines was estimated in nutrient solution containing 0 and 2 μM $AlCl_3$ (**Figure 5.2a**). Root growth in the absence of Al^{3+} was similar in all lines tested except Dayton which was approximately 20% lower. In 2 μM Al^{3+} solution, significant differences were detected with the transgenic lines Frd3_T2_40 and Frd3_T2_55 being 2.5-fold greater than the null line Null_T2_55 and Golden Promise. The relative root growth (RRG) was calculated as root growth in Al^{3+} as a percentage of growth in the control solution (**Figure 5.2b**). RRG in the control lines, Golden Promise and the line Null_T2_55, was inhibited to about 30% in 2 μM Al^{3+} nutrient solution, while the transgenic lines were inhibited to 67 % and 83% which was similar to Dayton.

5.3.3 Analysis of T3 plants

Since the Frd3_T2_40 and Frd3_T2_55 seed were limited, several plants were grown to generate T3 seed for further analysis. The null sister lines for each transgenic line were also grown on to provide T3 controls. About twenty seed from each of the newly harvested T3 lines, Frd3_T3_55 and Frd3_T3_40, were tested for hygromycin resistance using the leaf assay and for presence of the *Frd3* gene by PCR. Both families appeared to be homozygous since all of the seedlings showed resistance to hygromycin. Furthermore quantitative real-time PCR confirmed *Frd3*

Table 5.1 Segregtion of antibiotic resistance (hygromycin) in T2 families expressing *Frd3*. 1 cm leaf tips were cut from 5 days seedlings and inserted into MS medium with 200 mg/L hygromycin. The results were observed 6 days later. 'Line T2_', *Frd3* T2 generation; '40-' or '50-', *Frd3* T2 seed from the same T1 seedling. Homozygous lines and null lines were selected for further experiment. 'Homo', homozygous line; 'null', non-transgenic lines.

Lines	Resistant	Sensitive	Total	Selected
Line T2_40				
40-1	2	2	4	
40-2	1	0	1	
40-3	12	0	12	
40-4	5	1	6	
40-5	13	2	15	
40-6	14	3	17	
40-7	**11**	**0**	**11**	**homozygous**
40-8	2	0	2	
40-9	9	0	9	
40-10	5	1	6	
40-11	**0**	**21**	**21**	**null**
Line T2_55				
55-1	**0**	**21**	**21**	**null**
55-2	14	0	14	
55-3	**9**	**0**	**9**	**homozygous**
55-4	12	6	18	
55-5	7	3	10	
55-6	17	6	23	
55-7	7	0	7	
55-8	4	3	7	
55-9	0	9	9	
55-10	3	0	3	

expression in the root apices of the Frd3_T3_55 and Frd3_T3_40 lines while no expression could be detected in the null lines, Golden Promise or Dayton (**Figure 5.3**).

Citrate efflux from the root apices of the two T3 lines and nulls was measured with or without 50 μM Al^{3+} in the treatment solution. In the absence of Al^{3+} citrate efflux was only detected from the two transgenic T3 lines (**Figure 5.1c**). Whereas in the presence of $AlCl_3$, citrate was detected from the T3 transgenic lines and Dayton while Golden Promise and null lines showed none. No malate efflux was detected from any of the barley lines in the presence of Al^{3+} but it was detected from the wheat cultivar Carazinho (CZ) which was included as a positive control (**Figure 5.4**). The Al^{3+}-activated malate efflux in wheat is facilitated by the TaALMT1 transporter for which there is no functional homolog in barley.

Al^{3+} resistance of the two T3 homozygous lines was compared with nulls and control cultivars in hydroponic experiments by estimating relative root growth (RRG) following 5 d in nutrient solutions containing 0 μM, 1 μM, 2 μM and 4 μM $AlCl_3$. In control solution the root growth was similar for all the barley lines except for Dayton which, again, was about 20% lower (**Figure 5.5**). Relative root growth of the Frd3_T3_40 and Frd3_T3_55 lines was two to three-fold greater than Golden Promise and the null lines (Null_T3_40 and Null_T3_55) at all Al^{3+} concentrations. Frd3_T3_55 was not significantly different from the Al^{3+}-resistant cultivar Dayton in 1 μM and 4 μM $AlCl_3$ in these hydroponic solutions whereas the other homozygous line was less resistant.

5.3.4 Soil experiments

Growth of the T3 transgenic homozygous lines and control plants was also compared in short-term soil experiments using an acid soil with and without added lime. Relative changes in different plant, measurements in acid soil were compared to the limed soil as in previous chapters. Another transgenic barley line was included in the soil experiments for comparison. It was the same parental cultivar, Golden Promise, but expressing the wheat Al^{3+} resistance gene, *TaALMT1*.

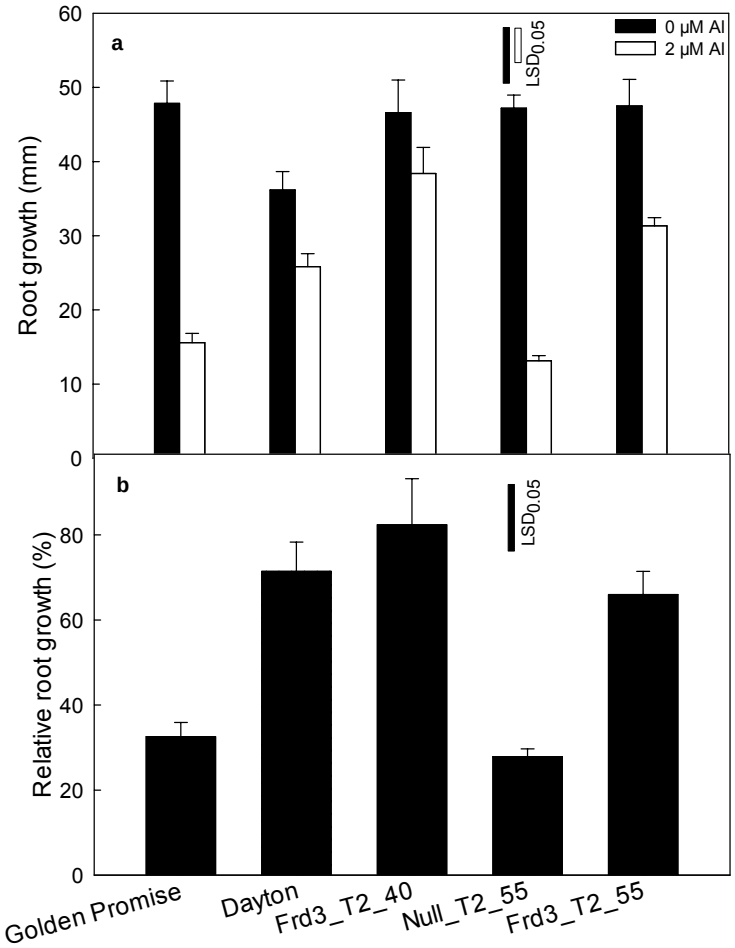


Figure 5.2 Al^{3+} resistance of T2 homozygous *Frd3* transgenic lines in hydroponic solution. Root growth was measured before placing seeds in hydoponics and after 4 days in nutrient solution containing 0 µM and 2 µM $AlCl_3$. Data show the mean of root growth and standard error (n=7). b. Relative root growth in the T2 homozygous *Frd3* transgenic lines. Al^{3+} resistance was estimated in T2 lines by measuring relative root growth in $AlCl_3$ compared with controls. Data show the mean of relative root growth and standard error (n=7). The bars represent the $LSD_{0.05}$.

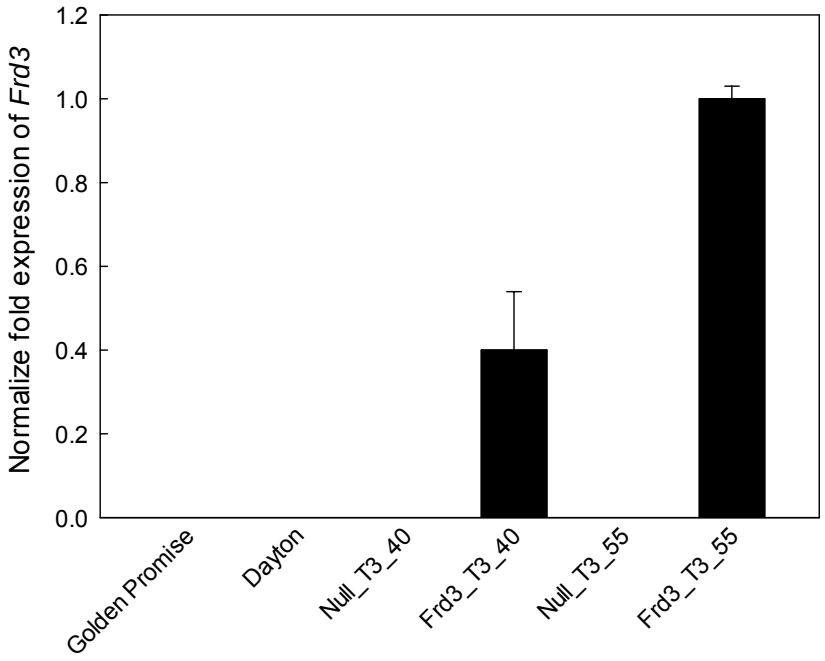


Figure 5.3 *Frd3* expression in transgenic homozygous T3 lines using qRT-PCR. Total RNA was extracted from eight root apices after 50 μM Al^{3+} treatment. Primer 4F and 4R (see **Table 2.5**) were used to amplify *Frd3*. Primers 7F and 7R (see **Table 2.5**) were used to amplify the endogenous *actin* gene. Data show the mean of expression level compared to actin with standard errors (n=4, 2 technical replicates of 2 biological replicates).

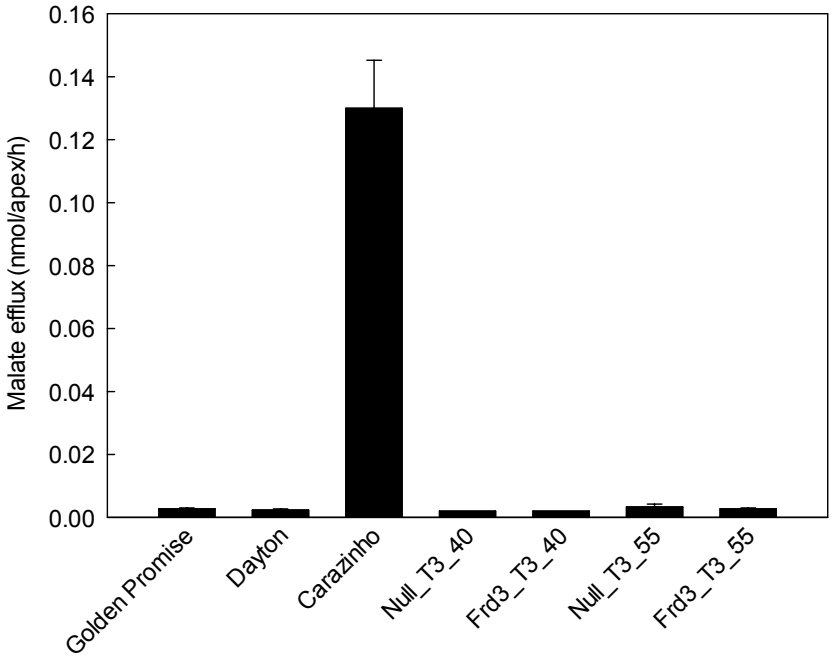


Figure 5.4 Malate efflux from root apices of T3 homozygous lines and control plants. Malate efflux was measured from excised root apices in the presence of 50 μM $AlCl_3$. Data show the means of malate efflux (nmol/apex/h) and standard errors (n=4).

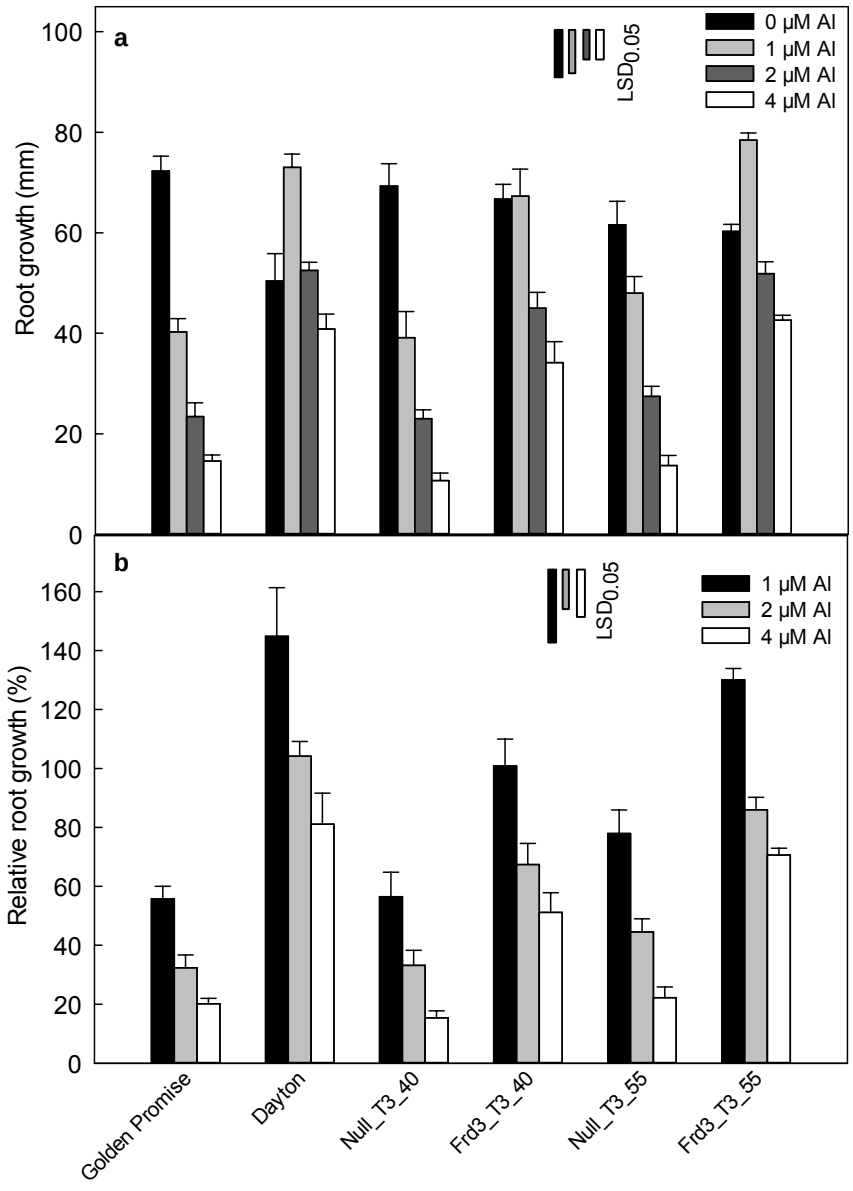


Figure 5.5 Al^{3+} resistance in hydroponic solution. a. Root growth in the T3 homozygous *Frd3* transgenic lines. Root growth was measured before placing seeds in hydoponics and after 4 days in nutrient solution containing 0 µM, 1 µM, 2 µM and 4 µM $AlCl_3$. Data show the mean of root growth and standard error (n=7). b. Relative root growth in the T3 homozygous *Frd3* transgenic lines. Al^{3+} resistance was estimated by measuring relative root growth in $AlCl_3$ compared with controls. Data show the mean of relative root growth and standard error (n=7). The bars represent the $LSD_{0.05}$.

Photographs of representative plants from these treatments prior to processing are shown in **Figure 5.6**. Shoot weight data and root weight data are presented in **Figure 5.7** and **Figure 5.8**. There were no consistent differences in shoot fresh weight and root fresh weight between transgenic lines and null lines when grown in acid soil or lime soil. Interestingly, most of barley lines have greater root fresh weight in acid soil than in limed soil (see Discussion). The relative shoot fresh weight and root fresh weight were calculated, and no differences between transgenics and their respective nulls were detected at $P_{0.05}$ level (**Figures 5.7b** and **5.8b**).

The root lengths showed more consistent differences between the transgenic and null plants. Results for the longest roots and the second-longest roots were similar (**Figures 5.9** and **5.10**). Relative longest root and second-longest roots in the two T3 transgenic lines were approximately 90% to 100% (acid soil relative to limed soil) which is significantly larger than the null lines at 60% to 70%. Golden Promise was also significantly smaller than the transgenic lines for the relative second-longest root length (**Figures 5.9b** and **5.10b**). RRG for Dayton was not significantly different from the two transgenic T3 lines but the relative longest root growth of the *TaALMT1* barley line was greater than one of the homozygous lines (Frd3_T3_55).

Similarly total root length showed consistent differences between the transgenic and non-transgenic lines (**Figure 5.11**). Relative total root length in the Frd3_T3_40 and Frd3_T3_55 transgenic lines was more than two-fold greater than their corresponding nulls and Golden Promise. It is unclear why the total root length of Frd3_7T3_40 was significantly lower than the other lines in this experiment. Dayton and the *TaALMT1*-barley had similar relative total root lengths to the transgenic lines.

Figure 5.6 Photographs of representative transgenic and control plants taken from the soils experiments prior to processing. a. Golden Promise (GP) grown in lime soil (left) and acid soil (right); b. Dayton grown in limed soil (left) and acid soil (right); c. Null_T3_55 grown in limed soil and acid soil; d. Homozygous *Frd3* line Frd3_T3_55 grown in limed soil (left) and acid soil (right).

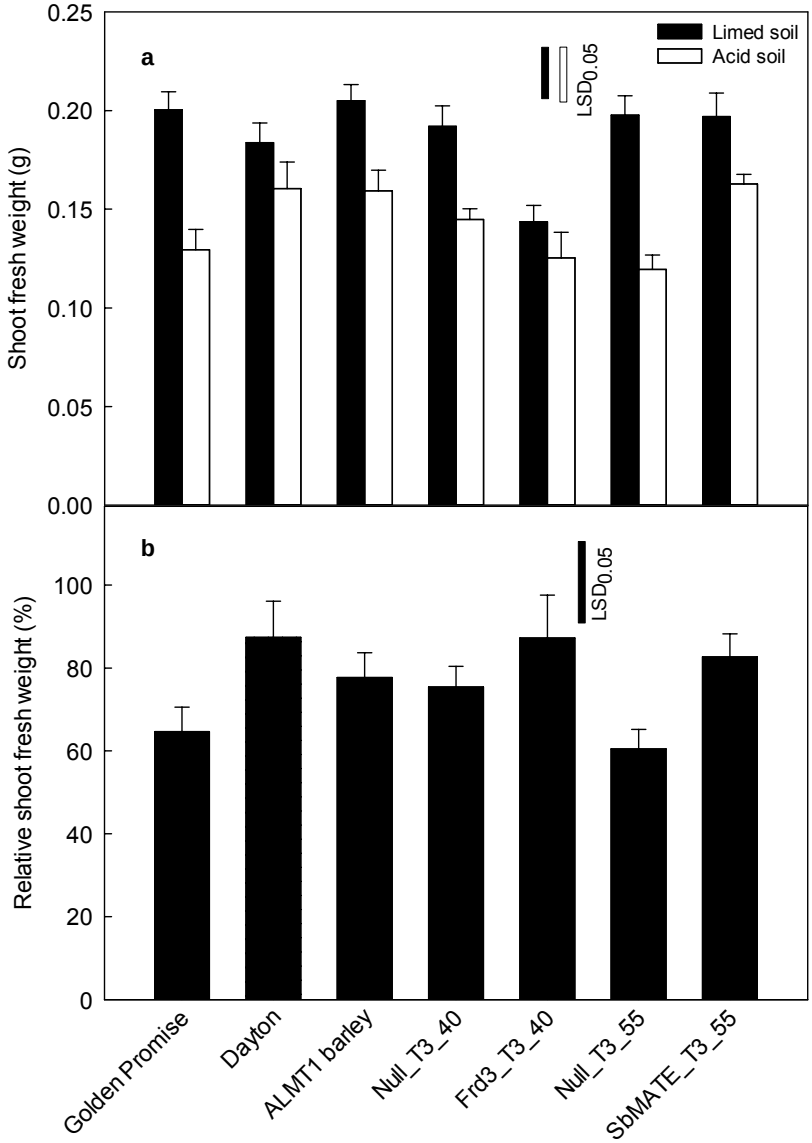


Figure 5.7 Shoot fresh weight in the T3 homozygous *Frd3* transgenic lines grown in acid soil and limed soil. A. Shoot fresh weight (g) with mean and standard error (n=6). b. The relative shoot fresh weight. Data show the mean of relative shoot fresh weight (shoot fresh weight in acid soil/shoot fresh weight in limed soil)x100 and standard errors (n=6). The bars represent the $LSD_{0.05}$.

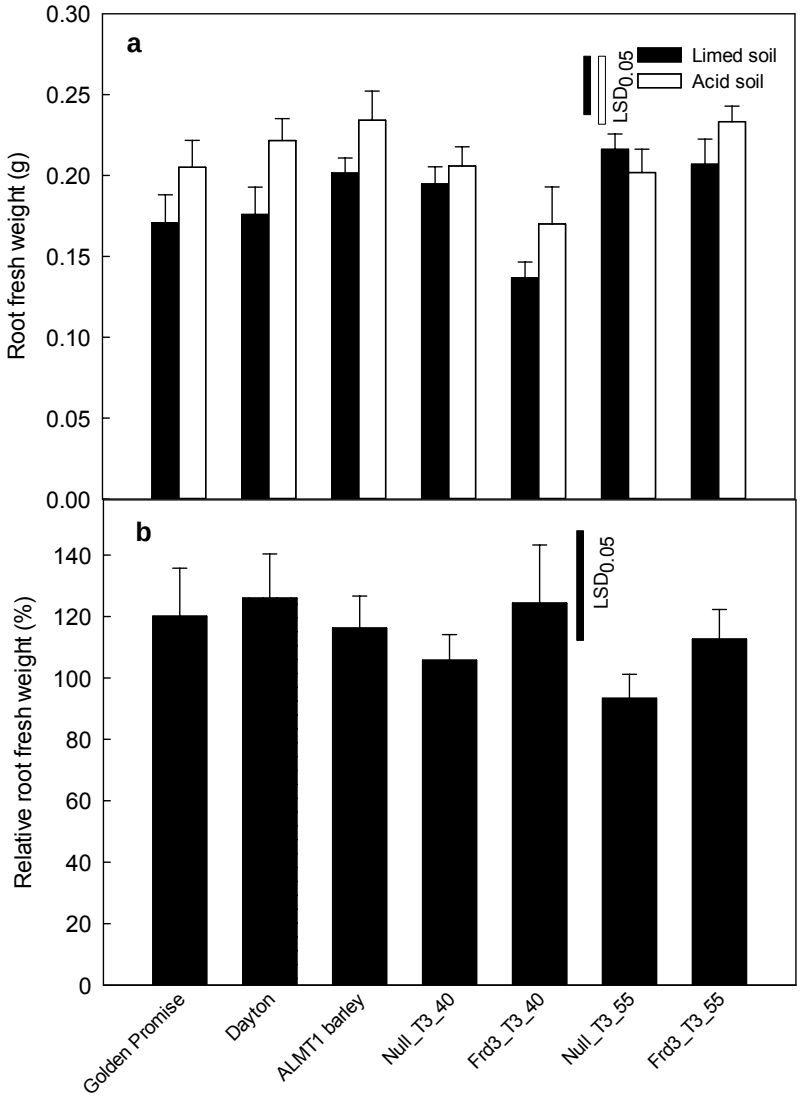


Figure 5.8 Root fresh weight in the T3 homozygous *Frd3* transgenic lines grown in soil. a. Root fresh weight of T3 homozygous *Frd3* transgenic lines on acid soil and limed soil. Data show the mean of root fresh weight (g) and standard error (n=6). b. The relative root fresh weight. Data show the mean of relative root fresh weight (root fresh weight in acid soil/root fresh weight in limed soil) x100 and standard error (n=6). The bars represent the $LSD_{0.05}$.

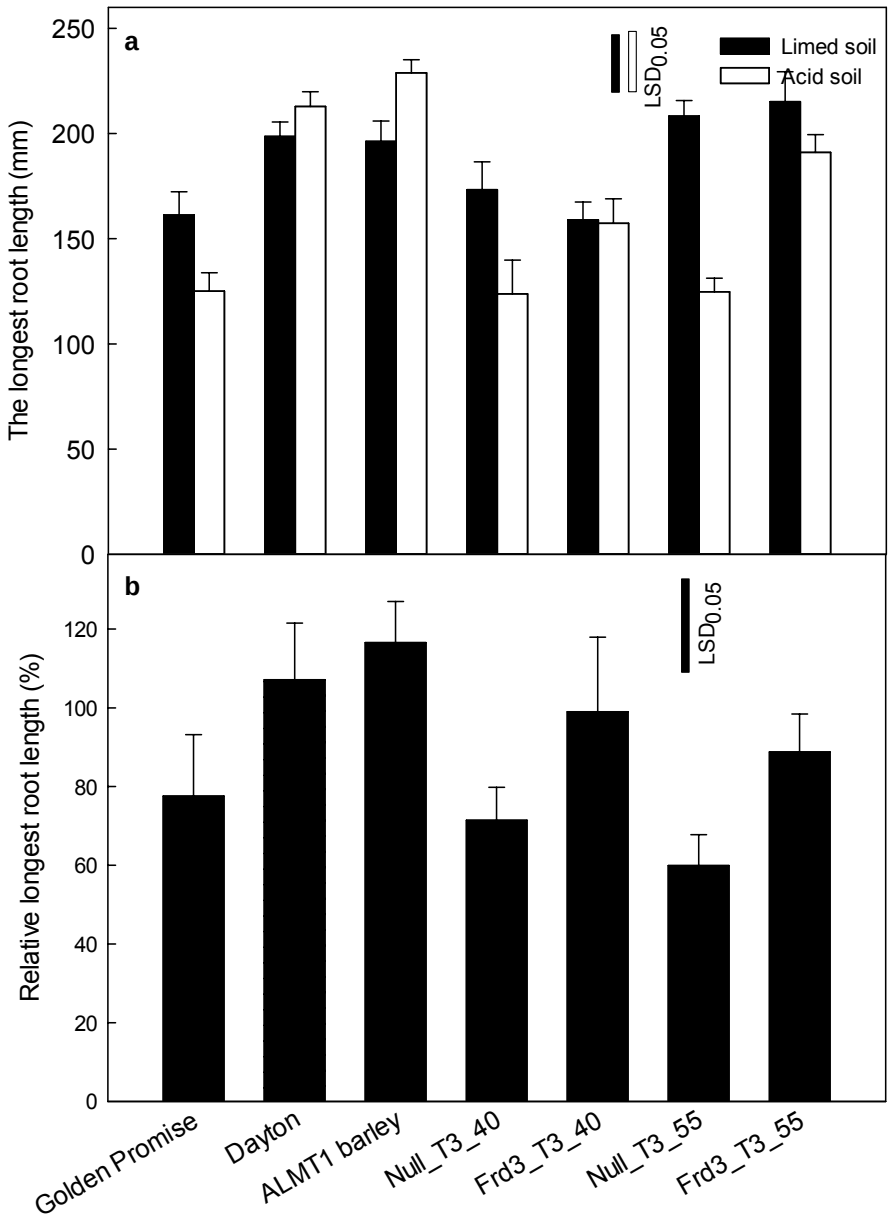


Figure 5.9 Longest root lengths in the T3 homozygous *Frd3* transgenic lines grown in soil. a. The longest root length of T3 homozygous *Frd3* transgenic lines on acid soil and limed soil. Root length were measured after 6 days. Data show the mean of the longest root length (mm) and standard error (n=6). b. Relative longest root length was estimated from the acid soil compared with the limed soil. Data show the mean and standard error (n=6). The bars represent the $LSD_{0.05}$.

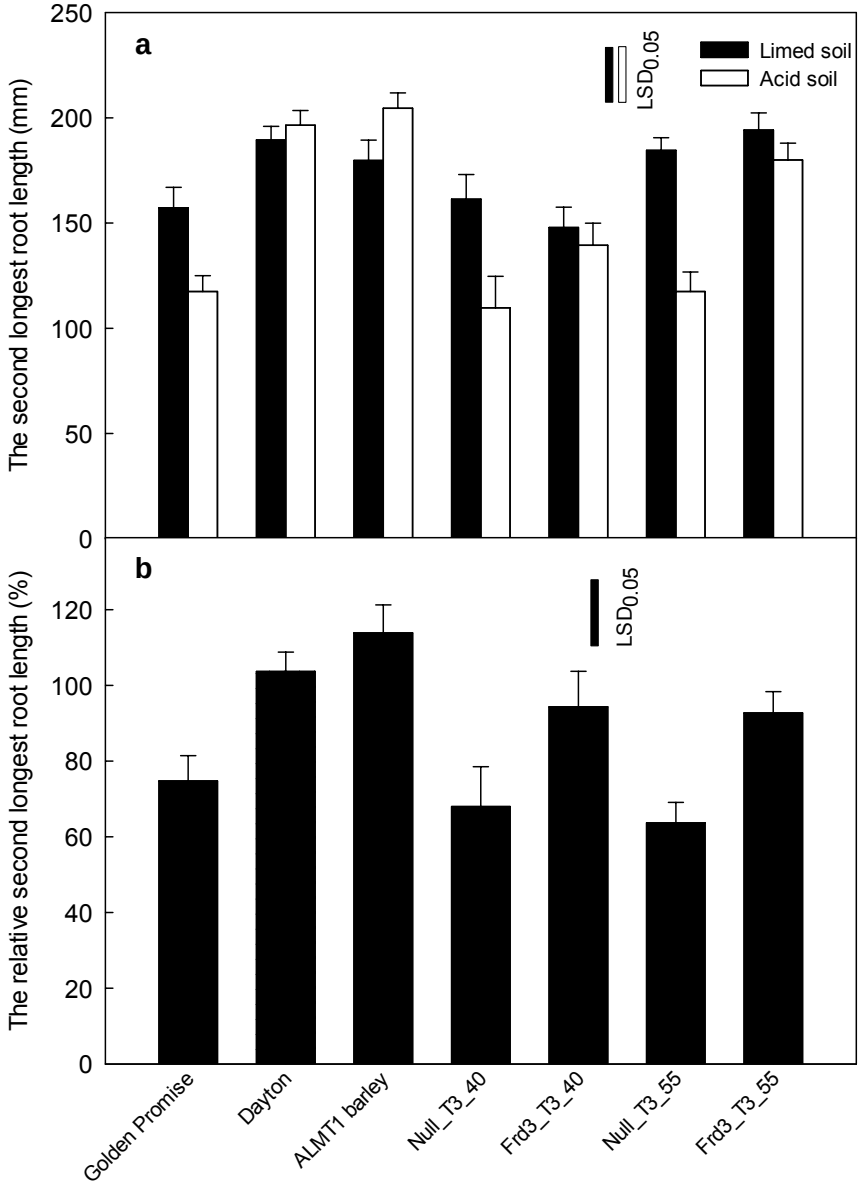


Figure 5.10 Length of the second-longest roots in the T3 homozygous *Frd3* transgenic lines grown in soil. a.The second-longest root length of T3 homozygous *Frd3* transgenic lines on acid soil and limed soil. Root length were measured after 6 days. Data show the mean of the second-longest root length (mm) and standard error (n=6). b. Relative secondest longest root length was estimated from the acid soil compared with the limed soil. Data show the mean and standard error (n=6). The bars represent the $LSD_{0.05}$.

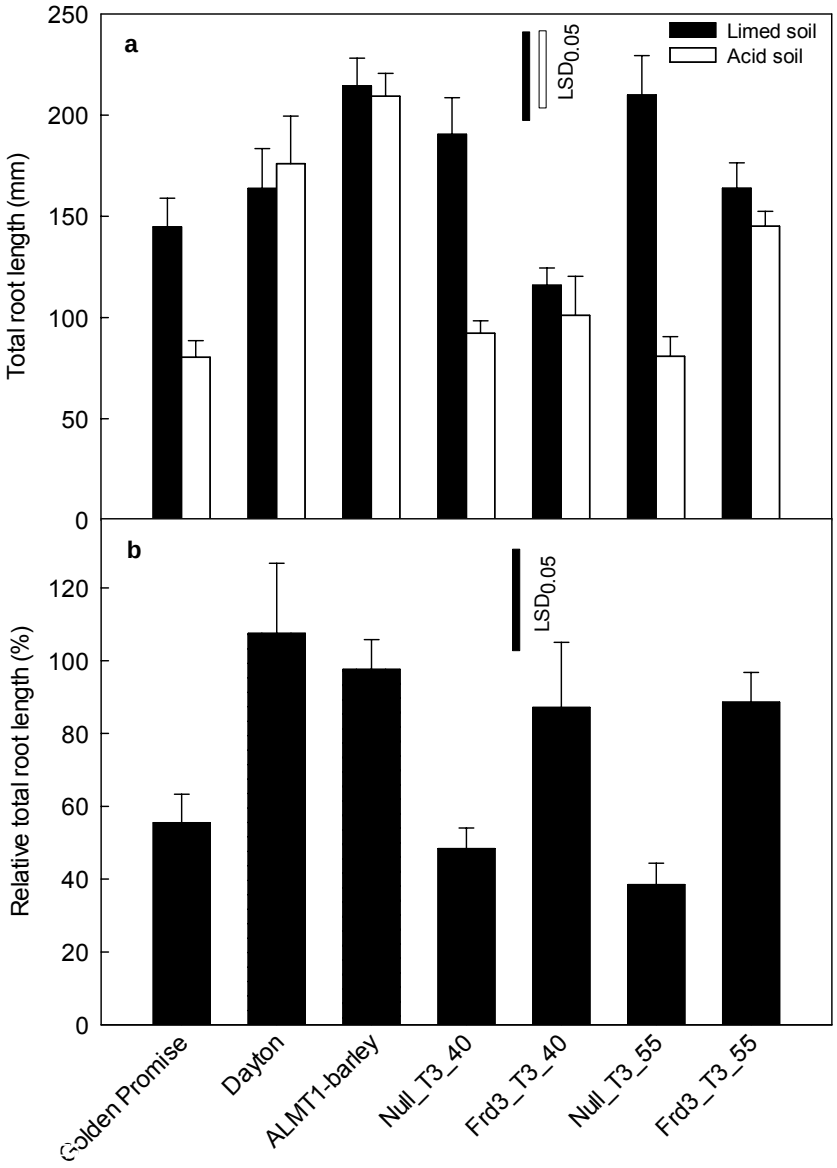


Figure 5.11 Total root length in the T3 homozygous *Frd3* transgenic lines grown in soil. a. Total root length after 6 days. Roots length was calculated by the WinRHIZO scanner. The data show the mean of total root length in acid soil and limed soil and standard error (n=6). b.The relative total root growth of the acid soil compared with limed soil. The data show the mean and standard error (n=6). The bars represent the $LSD_{0.05}$.

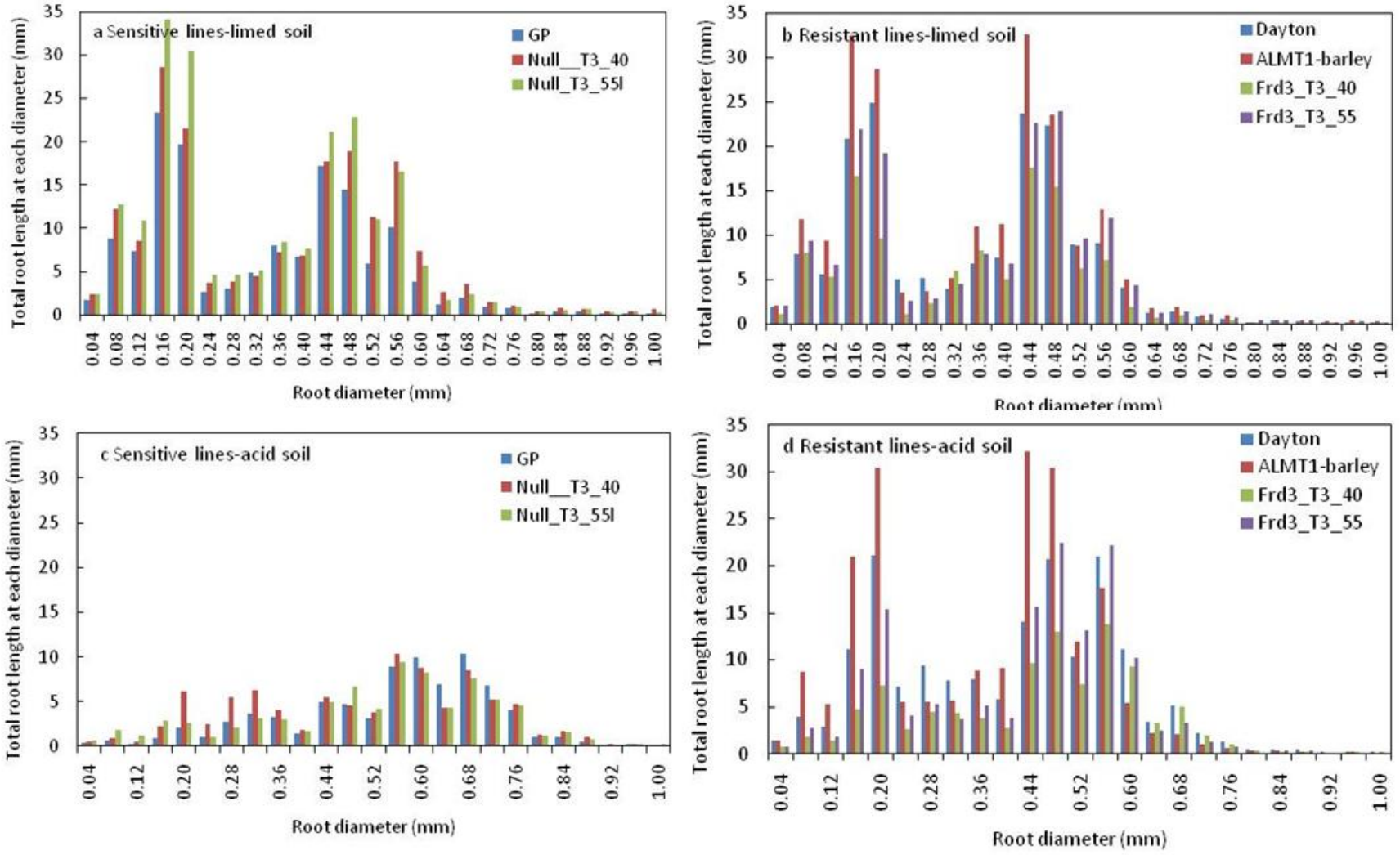


Figure 5.12 Distribution of root diameters of plants grown in soils

Figure 5.12 Distribution of root diameters of plants grown in soils. a. Total root length at each root diameter class from Al^{3+}-sensitive plants grown in limed soil. b. Total root length at each root diameter of Al^{3+}-resistant plants grown in limed soil. c. Total root length at each root diameter class of Al^{3+}-sensitive plants grown in acid soil. d. Total root length at each root diameter class of Al^{3+}-resistant plants grown in acid soil

The distribution of root diameters among the plants in each line were also measured using the WinRhizo software (**Figure 12**). As for the previous Chapters, the data were presented by grouping the Al^{3+}-resistant lines (Frd3_T3_40 and Frd3_T3_55, ALMT1-barley and Dayton) together and the Al^{3+}-sensitive lines (Null_T3_40 and Null_T3_55, Golden Promise) together based on the previous growth data. In limed soil, root diameters showed similar distributions in the Al^{3+}-resistant and sensitive lines with both showing peaks near 0.2 mm and 0.5 mm diameters (**Figures 5.12a** and **5.12b**). In acid soil, the Al^{3+}-resistant lines maintained a similar distribution of root diameters to the limed soil whereas the Al^{3+}-sensitive lines showed a marked decrease in the percentage of thinner roots (**Figures 5.12c** and **5.12d**). The total length of roots with the 0.2 mm diameters decreased while the total length of roots with diameters above 0.6 mm increased.

5.3.5 Elemental analysis

Since *Frd3* is involved with Fe nutrition in Arabidopsis the uptake and distribution of Fe was tested in barley plants expressing this gene. Two homozygous T3 lines, Frd3_T3_40 and Frd3_T3_55, one null line, Null_T3_40, and Golden Promise were grown for ten days in hydroponic solution containing 5 μM $FeCl_3$ or 100 μM $FeCl_3$. Plants growing in the low Fe treatment showed signs of Fe deficiency (pale leaves caused by interveinal chlorosis) which were not present in the high iron treatment. The complete set of elemental analysis from the shoot and roots is provided in Supplemental **Table S1** and a summary is presented in **Tables 5.2 and 5.3.** The results in **Tables 5.2 and 5.3** combine data from the two homozygous lines and from the null and parental lines for easy comparison. No statistical differences were detected in Fe concentrations between the transgenic and control plants but there were clear treatment effects. Shoot Fe concentration from the low Fe treatment was approximately 35 mg/kg which is half the concentration from the high Fe treatment and consistent with Fe deficiency in barley (**Table 5.3**). In roots, the Fe concentration was ~20-fold greater in high Fe compared to low Fe but, once again, no differences were detected between the transgenic and control plants. The low Fe treatment was also associated with 1.5 to 2-fold greater concentrations of Cu, Mn,

Pb, and Zn in shoots. In roots, low Fe induced >2-fold greater concentration of Cu but reduced concentrations of Mn and Pb. No significant differences were observed between the transgenic and control plants. The one possible exception was Cu concentration in roots under high Fe which was 63% higher in the transgenic lines compared to controls. However insufficient replicates were available to confirming this difference was statistically significant.

Table 5.2 Macroelement concentration in *Frd3* homozygous barley with iron treatment. Germinated barley seed (Wild-type cv. Golden Promise, Null line Null_Frd3_40 and two *Frd3* T3 lines 40 and 55) were grown in hydroponic solution containing 5 μM (deficient) and 100 μM (sufficient) Fe^{3+} for 15 days. Roots and shoots were harvested and dried and then conducted elemental analysis for one or two replicatess. Golden Promise and Null line were divided into the group 'GP+Null', and two Frd3 T3 lines were divided into the group 'Frd3_barley'. Data show the mean and standard error.

		Ca	K	Mg	Na	P	S
		%	%	%	%	%	%
Shoot	GP+Null	0.62±0.07	6.78±0.15	0.31±0.03	0.018±0.004	1.07±0.04	0.87±0.06
(iron deficient)	Frd3_barley	0.44±0.02	6.59±0.14	0.27±0.03	0.012±0.003	1.02±0.05	0.88±0.02
Shoot	GP+Null	0.70±0.02	7.13±0.16	0.25±0.01	0.026±0.003	0.95±0.03	0.48±0.03
(iron sufficient)	Frd3_barley	0.64±0.04	7.36±0.11	0.24±0.01	0.018±0.003	0.82±0.01	0.57±0.03
Root	GP+Null	0.24±0.01	4.88±0.15	0.08±0.00	0.042±0.001	0.89±0.01	0.79±0.02
(iron deficient)	Frd3_barley	0.21±0.00	5.00±0.60	0.07±0.00	0.043±0.004	0.99±0.14	0.86±0.14
Root	GP+Null	0.22±0.02	5.82±0.39	0.07±0.00	0.094±0.005	0.87±0.03	0.43±0.05
(iron sufficient)	Frd3_barley	0.25±0.01	6.32±0.02	0.06±0.00	0.065±0.004	0.88±0.03	0.50±0.02

Table 5.3 Microelement concentration of *Frd3* homozygous barley with iron treatment. Germinated barley seed (Wild-type cv. Golden Promise, Null line Null_Frd3_40 and two *Frd3* T3 lines 40 and 55) were grown in hydroponic solution containing 5 μM (deficient) and 100 μM (sufficient) Fe^{3+} for 15 days. Roots and shoots were harvested and dried and then conducted elemental analysis for one or two replicatess. Golden Promise and Null line were divided into the group 'GP+Null', and two Frd3 T3 lines were divided into the group 'Frd3_barley'. Data show the mean and standard error.

		Al	B	Cr	Cu	Fe	Mn	Pb	Ti	Zn
		mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg
Shoot	GP+Null	25.0±3.9	14.3±2.2	0.44±0.08	33.5±4.4	34.0±2.2	252.5±16.4	0.90±0.20	0.8±0.2	257.5±14.8
(iron deficient)	Frd3_barley	28.4±2.8	21.4±3.1	0.31±0.01	37.1±2.6	35.7±2.7	232.0±17.2	0.70±0.18	0.6±0.0	187.0±14.3
Shoot	GP+Null	25.5±1.1	15.4±1.6	0.23±0.03	24.3±0.9	68.4±2.7	114.0±7.1	0.53±0.10	0.4±0.1	84.6±7.5
(iron sufficient)	Frd3_barley	23.5±0.7	18.1±2.1	0.18±0.04	27.7±1.2	81.0±9.1	124.3±5.4	0.38±0.19	1.2±0.1	86.9±3.1
Root	GP+Null	100.3±1.9	9.0±0.4	0.68±0.03	1526±76	64.8±0.7	153.5±8.7	4.28±0.10	1.4±0.2	65.5±0.2
(iron deficient)	Frd3_barley	80.3±4.5	8.1±1.0	1.07±0.53	1913±673	67.2±4.0	166.3±14.4	3.80±0.30	1.3±0.1	65.6±7.8
Root	GP+Null	74.8±12.1	9.7±0.9	0.79±0.07	430±20	1260.0±80.0	293.5±36.5	26.50±1.50	1.7±0.1	81.1±8.3
(iron sufficient)	Frd3_barley	79.2±6.7	9.8±0.3	1.38±0.01	703±13	1066.0±94.0	365.5±2.5	23.00±2.00	1.8±0.0	100.1±2.9

5.4 Discussion

Frd3 is an *Arabidopsis* gene involved in iron nutrition. Its role is to deliver citrate to the xylem to aid the translocation of iron to the shoots and its absorption by leaf cells (Durrett et al., 2007). Since FRD3 facilitates citrate efflux from cells, its expression in *Arabidopsis* using the 35SCaMV promoter resulted in citrate efflux from the roots which enhanced the resistance of the transgenic plants to Al^{3+} stress (Durrett et al., 2007). In this part of the study *Frd3* was expressed in the Al^{3+}-sensitive cultivar Golden Promise to determine whether it can also enhance the Al^{3+} resistance of barley. *Frd3* expression in transgenic lines increased citrate efflux and improved root growth in Al^{3+} -toxic hydroponic treatments and in acid soil compared to the null controls and the parental cultivar. The phenotypes associated with *Frd3* expression were maintained in the T1, T2 and T3 generations. These experiments provide evidence that *Frd3* is capable of conferring Al^{3+} resistance to monocotyledons, a finding previously demonstrated for dicotyledons only.

Citrate efflux from the homozygous lines was detected in the presence and absence of Al^{3+} treatment. Even the apparent increase in efflux measured with Al^{3+} in Frd3_T3_55 was not statistically significant at $P_{0.05}$.This is similar to the findings for the over-expression of *Frd3* in *Arabidopsis* (Durrett et al., 2007) and demonstrates that FRD3 function is not activated by Al^{3+}. MATE proteins which are involved in conferring Al^{3+} resistance in other species, including HvAACT1 from barley, are activated by Al^{3+} (see Chapter 1). The only exception reported to date is TaMATE1 from wheat (Ryan et al., 2009, Ryan et al., 2011). The Al^{3+}-resistant cultivar of barley Dayton also showed citrate efflux but only in the presence of Al^{3+} as previously described (Furukawa et al., 2007, Wang et al., 2007).

Transgenic plants were evaluated in hydroponic experiments and in acid soil. The results indicate that relative root growth in hydroponic experiments and relative length of the longest root in soil-grown plants are the best parameters for estimating Al^{3+} resistance. Relative total root length is also satisfactory and this might be a more cost-effective way for estimating Al^{3+} resistance in large screens. Fresh shoot weight and fresh root weight did not correlate with Al^{3+} resistance and this is similar to previous reports (Carr and Ritchie, 1993, Muhling *et al.*, 1988). Interestingly, most of barley plants have greater root fresh weight in acid soil than

in limed soil. This is likely to be due to the tendency of roots to grow thicker in Al^{3+}. This is a response to Al^{3+} toxicity that has been reported previously (Carr and Ritchie, 1993). In some experiments the relative root length of Golden Promise was greater than the null lines (Null_T3_40 and Null_T3_55) and this might be related to the age of the Golden Promise seed and the conditions in which they were grown. These factors might affect the nutrient status of the seed and therefore the vigor of young seedlings.

The mineral content was measured in plants grown in hydroponic solution containing high and low Fe concentrations. After ten days growth plants from the low Fe treatment showed signs of Fe deficiency. No differences in shoot or root Fe concentration were detected between the *Frd3*-expressing plants and controls grown in high or low Fe treatment. Similarly, no differences in the concentration of the other major mineral elements were detected in the *Frd3*-expressing plants and controls grown in either Fe treatment. However Fe deficiency was associated with the greater concentrations of Cu, Mn, Pb and Zn in shoots in all lines and lower concentrations of Fe in the roots. This is consistent with previous results showing that there is considerable overlap in the mechanisms for acquiring and managing different mineral nutrients. This is especially true for the transition elements such as Fe, Mn, Cu, and Zn. For example, Treeby et al. (1989) found that root exudates of iron-deficient barley plants have a much higher capacity for micronutrient mobilization, for instance, Mn and Zn, when compared to iron-sufficient plants. Futhermore, under conditions of iron deficiency, the expression of *TOM1* and *HvTOM1* increased the efficiency of deoxymugineic acid (the primary of phytosiderophore) secretion in rice and barley roots, respectively, by an enhanced expression of these genes (Nozoye *et al.*, 2011). Moreover, the deoxymugineic acid can also bind other metals, such as Cu (Gries *et al.*, 1998) and Zn (Erenoglu *et al.*, 2000). Internal nicotianamine levels (Douchkov *et al.*, 2005) elevate Fe, Zn, Ni and Mn in shoots indicating that these metal ions are bound by similar compounds as they move from the root to the shoot. Therefore, cells need to balance the demands of Fe nutrition with the risks of inadvertently absorbing other non-limiting minerals to excess.

These results demonstrate that heterologous expression of the *Arabidopsis* gene *Frd3* can increase the Al^{3+} resistance of barley by increasing the constitutive efflux of citrate from the roots.

CHAPTER 6 Direct comparison of the three *MATE* genes

6.1 Introduction

The previous chapters in this thesis described how *HvAACT1* from barley, *SbMATE* from sorghum and *Frd3* from *Arabidopsis* were over-expressed in the Al^{3+}-sensitive barley cultivar Golden Promise. Although they all belong to *MATE* gene family they do differ in their functions. For instance, in contrast to HvAACT1 and SbMATE, the Frd3 protein does not need to be activated by Al^{3+} to release citrate. The resulting transgenic plants from these transformation events were evaluated for gene expression, citrate efflux and Al^{3+} resistance in hydroponics and in soils. Since these experiments were all performed separately, the relative effectiveness of these genes to confer Al^{3+} resistance was unclear. To determine which of these genes confers the greatest level of Al^{3+} resistance, the best performing T2 or T3 line from each set of transgenics was selected and evaluated under the same conditions. The objective of this chapter was to compare the transgenic plants expressing the various *MATE* genes under the same conditions and at the same time. The relative root growth was also compared among transgenic *MATE* barley plant and *TaALMT1* barely plant.

6.2 Materials and methods

The best performing lines from each gene construct with regard to Al^{3+} resistance they confer were selected. These included HvAACT1_T2_51, Frd3_T3_55 and SbMATE_T3_133. Two null lines, Null_T2_51 and Null_T3_55 were also chosen. Null_T2_51 is the sister lines of HvAACT1_T2_51 and Null_T3_55 is the sister line of Frd3_T3_55. Seedlings from these lines were grown together in hydroponic treatments with the parental line Golden Promise and the Al^{3+}-resistant cultivar Dayton. TaALMT1 and these MATE transgenic barley lines were also grown for five days in hydroponic solutions with 2, 5 and 10 (or 15) μM $AlCl_3$ at pH 4.3 or 3.7. Root growth was estimated by measuring the root length before and after the growth experiment. Citrate efflux, relative expression level and Al^{3+} resistance of these lines were evaluated according to procedures described in Chapter 2. Since the expression level of different genes might be determined by the specific primers

and by the size of the amplicons, we accounted for the different efficiencies of the primers in the qRT-PCR for each *MATE* gene and *actin* when comparing their expression levels. The amplification efficiencies of these genes together with the endogenous gene *actin* were calculated using the software LinRegPCR (Ruijter *et al.*, 2009). Implemented was exported from C1000^{TM} Thermal Cycler (Bio-RAD, USA). After the data was read into LinRegPCR, the PCR efficiency (E), fractional number of cycles needed to reach the fluorescence threshold (C_q) and the observed fulorescence data (N_q) can be obtained from the program. According the equation, N_0 (the starting concentration) = N_q/E^{Cq} (Ruijter et al., 2009), the starting concentration of the genes can be calculated and the relative expression level of transgenes to *actin* are determined.

6.3 Results

Relative expression level of each transgene was calculated relative to the reference gene *actin* (**Figure 6.1**). These calculations accounted for primer efficiency which allowed the expression to be compared directly even though they amplify regions of different genes. Expression of the endogenous *HvAACT1* gene in the cultivar Dayton was the lowest among any of the transgenic and non-transgenic lines. Expression level of *HvAACT1* in the transgenic line HvAACT1_T2_51 was about 1.5-fold greater than *HvAACT1* in Dayton. Expression of *Frd3* in Frd3_T3_55 transgenic line was two-fold greater than *HvAACT1* in Dayton and expression of *SbMATE* in SbMATE_T3_133 was eight-fold greater. As expected no expression of *HvAACT1* was not detected in Golden Promise, no expression of *HvAACT1* was detected in Null_T2_51 and no expression of *Frd3* was detected in Null_T3_55.

Citrate efflux from the null lines and Golden Promise was low and considered background. Even though the excised tissues are washed, some leakage may still occur from the cut surfaces. Among these three transgenic lines, homozygous line Frd3_T3_55 had the greatest citrate efflux from root apices. This was statistically different from SbMATE_T3_133 and HvAACT1_T2_51 but not from Dayton (**Figure 6.2**). SbMATE_T3_133 was the same as Dayton and HvAACT1_T2_51 was about 50% of Dayton.

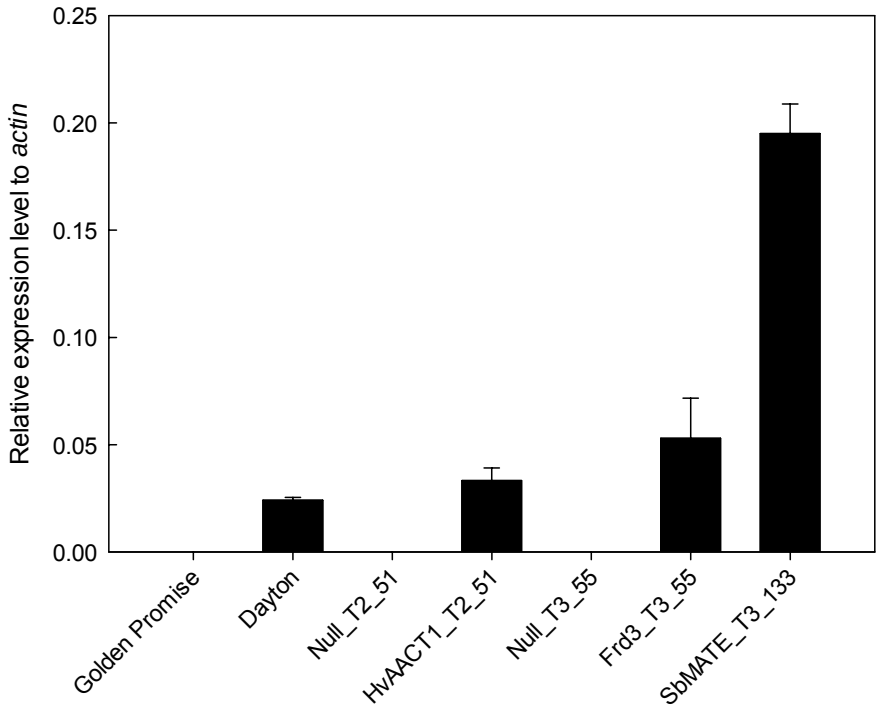
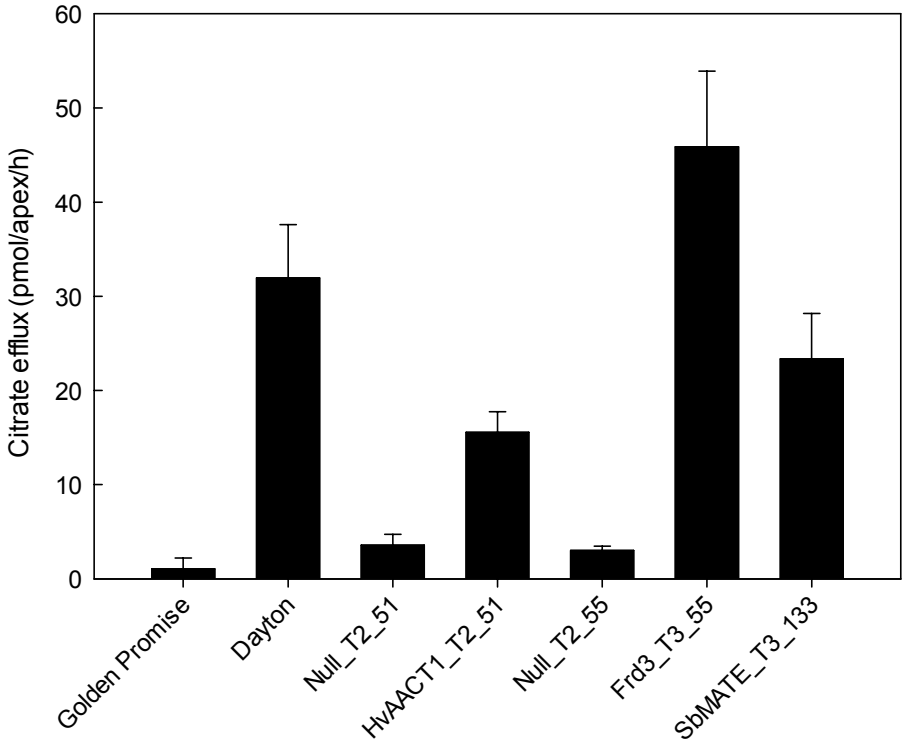


Figure 6.1 *MATE* expression level relative to the reference gene *actin*.These calculations account for primer efficiency so the results can be compared directly. The genotypes include the parental cultivar Golden Promise (Al^{3+}-sensitive); Dayton, positive control barley (Al^{3+}- resistant); HvAACT1_T2_51 (T2 homozygous line expressing *HvAACT1*); Frd3_T3_55 (T3 homozygous transgenic line expressing *Frd3*); SbMATE_T3_133 (T3 homozygous line expressing *SbMATE*); Null_T2_51 (null sister line to HvAACT1_T2_51); and Null_T3_55 (null sister line to Frd3_T3_55). Data show the mean and standard error of relative expression level (n=4 biological replicates).



Figrue 6.2 Citrate efflux from excised root apices. Genotypes are the same as in Figure 6.1. Data show the mean and standard error of citrate efflux (n=4 biological replicates).

The Al^{3+} resistance of these lines was evaluated in hydroponic solutions containing 0, 1, 2 and 4 μM $AlCl_3$. Dayton grew shorter roots in control conditions (~40 mm) compared to the 70 mm in the other lines (**Figure 6.3a**). First, relative root growth (RRG) of the resistant cultivar Dayton was significantly greater than Golden Promise at all Al^{3+} treatments (**Figure 6.3b**). Similarly RRG of the transgenic line Frd3_T3_55 was also significantly greater than its null sister line at all Al^{3+} treatments. Transgenic line HvAACT1_T2_51 was significantly greater than its null sister lines at 1 μM Al^{3+} but not at 2 and 4 μM Al^{3+} at the 0.05 level, and significantly greater than wild-type Golden Promise at all concentrations. RRG for Golden Promise and the two null lines was 40 to 50% at 1 μM Al^{3+} whereas it remained near 100% for the three transgenic lines and Dayton (**Figure 6.3b**). At 4 μM $AlCl_3$, RRG of Dayton and Frd3_T3_55 was 55 to 60% which was significantly greater than all other lines.

To compare Al^{3+} resistance between transgenic *TaALMT1* and *MATE* barley lines, they were grown in hydroponic solutions containing 0, 2, 5 and 15 μM $AlCl_3$ at pH 4.3. In control solution, they were no difference in root growth among Golden Promise and transgenic *TaALMT1* and *MATE* (*SbMATE*, *HvAACT1* and *Frd3*) lines. When Al^{3+} was applied to the solution, all of the lines were inhibited, while TaALMT1-barley was less inhibited than MATE transgenic and Golden Promise lines (**Figure 6.4a**). Their relative root growth (RRG) were calculated and displayed in **Figure 6.4b**. TaALMT1-barley showed the greatest RRG in each different concentration of Al^{3+} treatments. In $AlCl_3$ solutions, all of transgenic *MATE* lines and Golden Promise showed decreasing RRG from 20%~55% to 10%~20% when Al^{3+} concentration increases from 2 μM to 15 μM. Among MATE lines, T3_SbMATE_133 displayed the best performance in Al^{3+} resistance. For TaALMT1-barley, the RRG was about 80% in 2 μM Al^{3+} solution which was about two-fold greater than transgenic *MATE* lines. When Al^{3+} concentration increased from 5 μM to 15 μM, unlike other lines the RRG for TaALMT1-barley increased from 40% to 58%.

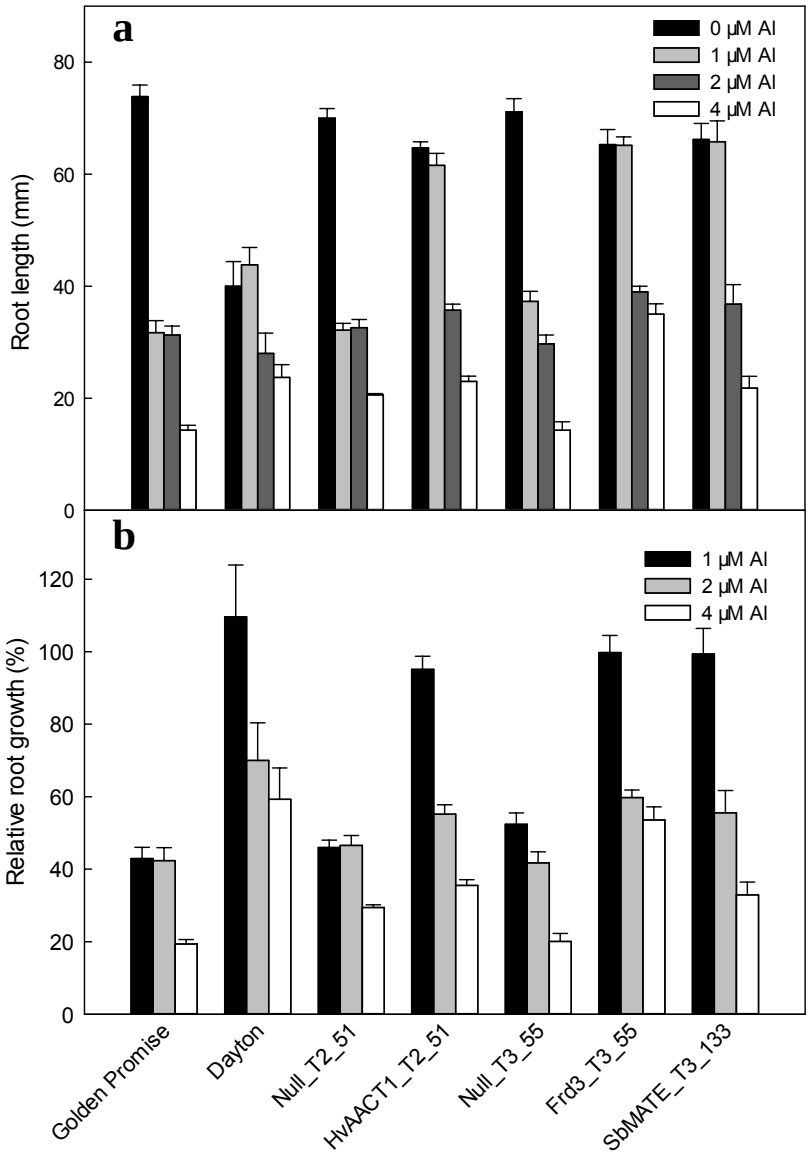


Figure 6.3 Al^{3+} resistance of the barley lines in hydroponic culture. a. Genotypes described in Figure 6.1 were grown in hydroponic solutions containing 0, 1, 2 and 4 µM $AlCl_3$ and net root growth measured after 4 days. b. Al^{3+} resistance was estimated from relative root growth (RRG) which is calculated as root growth in 1, 2 and 4 µM $AlCl_3$ compared to the control solution. Data show the mean and standard error of relative root growth (%). $LSD_{0.05}$ of RRG are 18, 13 and 10 at 1, 2 and 4 µM $AlCl_3$ respectively. The bars represent the $LSD_{0.05}$.

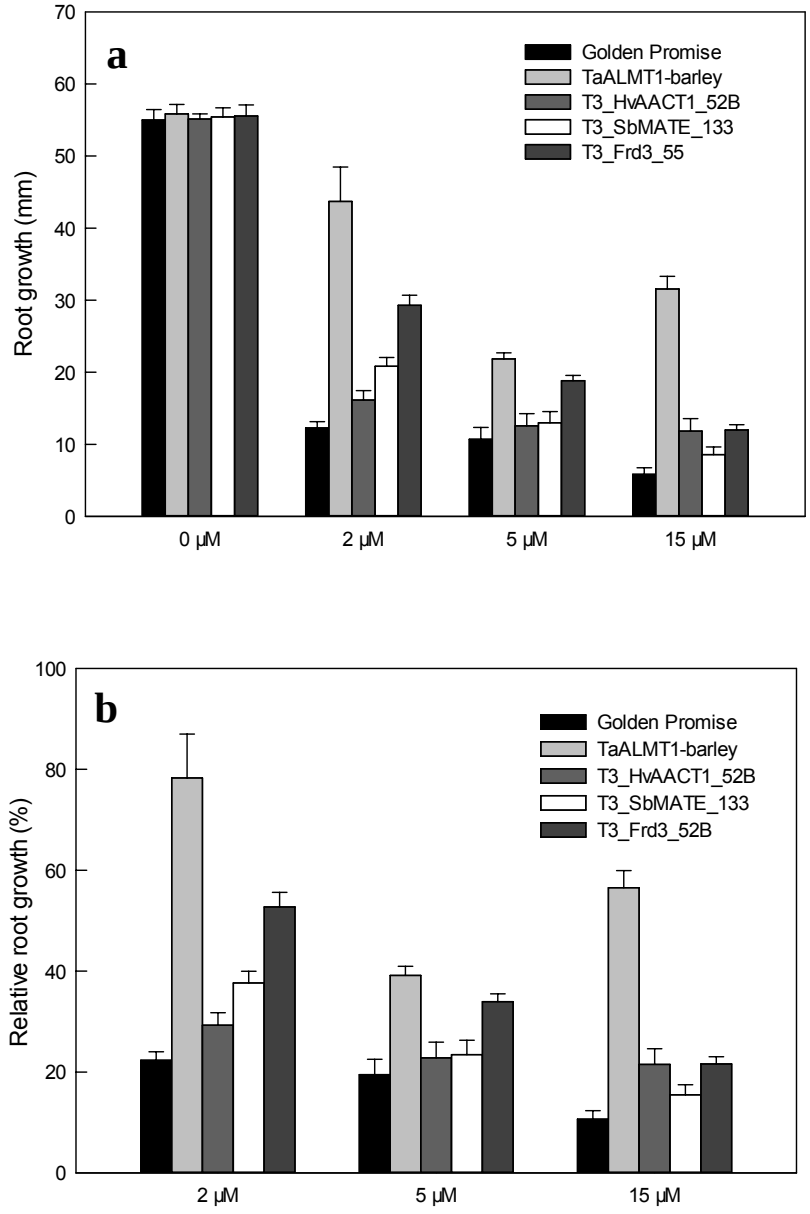


Figure 6.4 Al^{3+} resistance comparisons among transgenic barley lines carrying *TaALMT1* and *MATE* (*HvAACT1*, *SbMATE* and *Frd3*) genes. Seven seedlings of each line were grown in hydroponic solutions containing 0, 2, 5 and 15 µM $AlCl_3$ with pH 4.3, respectively. The root length was measured before placed in solutions and after five days. a. Net root growth measured after 5 days. Genotypes were described in Figure 6.1. $LSD_{0.05}$ of root growth are 3.4, 6.5, 3.7 and 3.6 at 0, 2, 5 and 15 µM $AlCl_3$, respectively. b. Al^{3+} resistance was estimated from relative root growth. Data show the mean and standard error. $LSD_{0.05}$ of relative root growth are 14, 8 and 7.7 at 2, 5 and 15 µM $AlCl_3$, respectively.

The Al3+ resistance of these lines was also compared in more acidic condition of pH 3.7 (**Figure 6.5**). The root growth of Golden Promise, TaALMT1-barley, T3_SbMATE_133 and T3_Frd3_55 were measured. The root growth of Golden Promise decreased from ~28 mm to ~10 mm when Al^{3+} was applied from 0 to 10 µM. In acid hydroponic solution without Al^{3+}, the root growth of all the lines was ~25-35 mm. When 2 µM Al^{3+} was added to the solution, root growth of *TaALMT1* and two *MATE* lines increased to ~60-70 mm. After Al^{3+} concentration raised to 5 µM and 10 µM, the root length of TaALMT1-barley dropped to 38 mm in 5 µM Al^{3+} solution and ~50 mm in 15 µM Al^{3+} solutions, while the other two transgenic MATE lines decreased to ~28-35 mm. T3_SbMATE_133 showed better root growth in acidic or with 2 µM Al^{3+} conditions than TaALMT1-barley, while in higher Al^{3+} concentration, TaALMT1-barley exhibited better.

The seed from transgenic *TaALMT1* and *MATE* barley lines grown in normal soil were conducted elemental analysis. No significant differences were found between transgenic barley lines and parental wild-type line (Table S2).

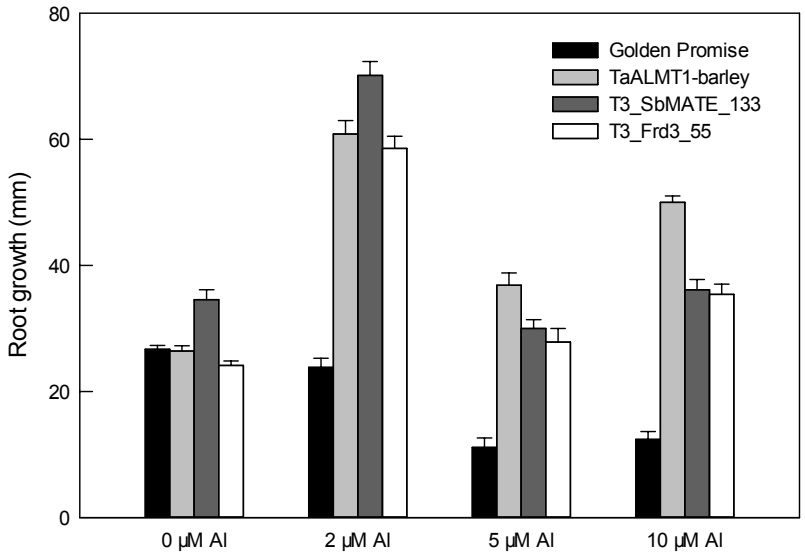


Figure 6.5 Al^{3+} and acid resistance comparisons between transgenic barley lines carrying *TaALMT1* and *MATE* (*HvAACT1*, *SbMATE* and *Frd3*) genes. Seven seedlings of each line were grown in hydroponic solutions containing 0, 2, 5 and 10 µM $AlCl_3$ with pH 3.7, respectively. The root length was measured before placed in solutions and after five days. Genotypes were described in Figure 6.1. $LSD_{0.05}$ of root growth are 3.3, 5.3, 4.2 and 4.5 at 0, 2, 5 and 10 µM $AlCl_3$, respectively. Data show the mean of root growth and standard error.

6.4 Discussion

This chapter directly compared the three different *MATE* genes side by side to determine whether one was more effective than the others at increasing the Al^{3+} resistance of barley. Based on these results *Frd3* appears to confer a slightly greater level of resistance in hydroponics compared to *SbMATE* and *HvAACT1*. However it is interesting that all three genes performed very similarly despite large differences in their relative expression and smaller but significant differences in the citrate efflux they generated.

After allowing for differences in primer efficiency the expression level of *MATE* genes was not correlated with citrate efflux. For instance, *SbMATE* expression in SbMATE_T3_133 was about eight-fold greater than in Dayton yet the citrate efflux from SbMATE_T3_133 was smaller than Dayton. Similarly the expression level of *Frd3* in Frd3_T3_55 was about 25% the expression of *SbMATE* in SbMATE_T3_133, but citrate efflux from Frd3_T3_55 was two-fold greater. The correlation between the expression level of these genes and citrate efflux was poor.

Several explanations are possible to explain this lack of correlation between expression and citrate efflux. Firstly, gene expression may not be directly correlated with the protein level. Alternatively the three MATE proteins may be regulated differently by posttranslational modifications or perhaps there are innate differences in the efficiency with which these MATE proteins transport citrate out of the cell. This is difficult to assess without direct comparisons of citrate efflux and protein level but based on the current data *SbMATE* would appear to be the least efficient. This gene shows the greatest transcript levels but generates only moderate levels of citrate efflux. It is also possible that efficient citrate efflux requires expression of the *MATE* genes in specific cells or in specific tissues. Furukawa et al. (2007) reported that the expression level of *HvAACT1* was 2-3 fold higher in the basal root tips than the root tips, and citrate efflux may be largely restricted in that region also. In this experiment, expression of *HvAACT1*, *SbMATE* and *Frd3* was driven by the constitutive promoter ubiquitin which expresses these genes throughout the plant. Therefore the correlation between citrate efflux and expression of the endogenous *HvAACT1* gene in Dayton may be different to the relationships found with the other *MATE*s in transgenic lines where the transgene is expressed in all root cells. Lastly,

MATE expression might not be the major determinant of citrate efflux. Membrane-bound transport proteins are required to facilitate citrate transport across the plasma membrane but it is possible that after a certain level of expression it is the supply of citrate, not the number of transporters, that limits efflux or citrate and other organic anions.

ALMT1 and *MATE* transgenic barley lines were compared in Al^{3+} and acid conditions. Both of them are sensitive to acid solution. Under Al^{3+} stress, TaALMT1-barley is more resistant than transgenic *HvAACT1*, *SbMATE* and *Frd3* lines. Relative root growth of TaALMT1-barley was smaller in 5 μM Al^{3+} solution than in 15 μM Al^{3+} solution. It is possible that Al^{3+} replaced some of H^{+} on the roots surface in 5 μM Al^{3+} solution, so both acidic stress and Al^{3+} stress impose on TaALMT1-barley.

In conclusion, although the transcript levels of the three *MATE* genes, *HvAACT1*, *Frd3* and *SbMATE*, differed in the transgenic plants all conferred a similar degree of Al^{3+} resistance. Surprisingly this level of resistance was similar to, but never greater than, that found in the Al^{3+} resistant cultivar Dayton.

CHAPTER 7 General discussions and conclusions

Enhancing plant resistance to Al^{3+} stress by over-expression of citrate or malate transporters is a powerful and promising approach (Delhaize et al., 2004, Furukawa et al., 2007, Magalhaes et al., 2007, Pereira et al., 2010). For instance the *TaALMT1* gene from wheat encodes an Al^{3+} activated malate channel and functions as the major Al^{3+} resistance gene in wheat. When this gene was expressed in tobacco suspension cells (Sasaki et al., 2004), barley (Delhaize et al., 2004) and *Arabidopsis* (Ryan et al., 2010) or over-expressed in wheat (Pereira et al., 2010), it conferred greater malate efflux and increased resistance to Al^{3+} stress. This study describes the expression of three members of the *MATE* gene family (*HvAACT1*, *SbMATE* and *Frd3*) in an Al^{3+} sensitive barley cv. Golden Promise. The transgenic plants expressing these genes showed similar phenotypes: increased citrate release from root apices and an increase in Al^{3+} resistance in both hydroponic culture and acid soil. These increases in citrate efflux and Al^{3+} resistance were not observed in the control plants which were the null segregant lines and parental cultivar Golden Promise. This is the first report demonstrating that over-expression of *MATE* genes can enhance the Al^{3+} resistance of this cereal species.

Interestingly the transgenic barley plants expressing *TaALMT1*, which were included as positive controls in the soil experiments, displayed similar levels of Al^{3+} resistance to the transgenic lines expressing the *MATE* genes. At first glance this result is surprising, since plants expressing *TaALMT1* release malate from roots while the MATEs facilitate more citrate from their roots and citrate binds with Al^{3+} more strongly than malate does. The formation constant between $citrate^{3-}$ is 9.6 compared to 5.4 for $malate^{2-}$ and Al^{3+} (Ryan et al., 2001). However the similarity in the phenotypes of these two groups is related to the fact that efflux of citrate from the plants expressing the *MATE* genes is about 10 to 20-fold less the malate efflux from plants expressing *TaALMT1* (Delhaize et al., 2004) and this compensates for the lower formation constant.

The transgenic lines generated in this study should be used for yield trials in the field. However experiments with genetically modified plants are difficult to perform in the field and beyond the scope of a PhD project since the relevant laws are very

restrictive. Furthermore, such experiments, when possible, should be conducted in at least three environments for three years in acid soil and limed soil, in order to calculate environmental effect on yield.

The correlation between expression level of transgenes and citrate efflux was poor (**Figures 4.1, 6.1 and 6.2**). Perhaps expression of these MATE genes was regulated post-transcriptionally such that the amount of protein was not correlated with the RNA expression level. Citrate may be released from specific cells in the root tips region, so the correlation between expression level and citrate efflux is restricted in the specific tissue. Alternatively, perhaps efficiency difference in transporting citrate out of the cells per unit protein exists in these three MATE genes. It is also possible that the supply of citrate within the barley roots becomes the limiting factor in some cases. This demonstrates that the expression level of MATE genes from the entire root apices is not a necessary parameter for transgenic lines with great citrate efflux.

Interestingly, the Al^{3+} resistance of transgenic plants expressing *HvAACT1*, *Frd3* and *SbMATE* did not exceed the Al^{3+}-resistant wild-type cv. Dayton. The transgenic lines could be used to cross the *MATE* genes into the Dayton background to determine whether the effect of the transgenes and the natural resistance of Dayton are additive. These crosses were initiated in this project but the crosses failed.

Since Al^{3+} resistance of barley plants was assessed in hydroponics and in soil some conclusions can be made regarding screening methodologies. Rather than waiting for yield data from field trials to rank barley plants for Al^{3+} resistance, short-term screens in hydroponics or pots experiments could save time and energy. Nutrient solution is an easy and fast way to assess Al^{3+} resistance. Transgenic plants displayed great Al^{3+} resistance in hydroponic solutions. The relative root growth (RRG) of transgenic plants was about 100% in nutrient solutions containing 1 μM $AlCl_3$, whereas 50% for wild-type and null lines (**Figures 3.5**, **4.4**, **5.5** and **6.3**). It further supports that *HvAACT1*, *Frd3* and *SbMATE* play a role in Al^{3+} resistance in transgenic barley plants. This is consistent with previous reports. When they were transferred into tobacco or *Arabidopsis*, an increased Al^{3+} resistance was observed in their transgenic plants (Durrett et al., 2007, Furukawa et al., 2007, Magalhaes et al., 2007). The parameters assessed in pot experiments with acid or limed soil were

fresh root weight, fresh shoot weight, the longest root length, the second longest root length and total root length and the distribution of root diameters. Length of the longest and second longest root and total root length were the most reliable parameters to differentiate Al^{3+}-resistant and Al^{3+}-sensitive plants – especially when the lines were compared with their own performance in limed soil so that relative longest root etc can be to generated. Measurements of total root length are too time-consuming and costly, so root length is still the best assessment for Al^{3+} resistance in acid soil. Generally shoot parameters are not significant between transgenic and non-transgenic lines since the root apices are the most sensitive region, and in this short time (6 d) experiment, difference in shoot fresh weight is not obvious. While not a convenient screen the distribution of root diameter, especially for *SbMATE* and *Frd3* transgenic barley was very diagnostic for Al^{3+} stress. In limed soil, there was no difference in distribution of root diameter between transgenic (Al^{3+}-resistant) and null (Al^{3+}-sensitive) lines. Both of them have too frequency peaks (**Figures 4.12a, 4.12b, 5.12a and 5.12b**), which likely represent lateral roots and main roots. In acid soils, lateral roots disappeared in Al^{3+}-sensitive lines and length of main roots decreased (**Figures 4.12c and 5.12c**), while there are the two peaks are maintained in the Al^{3+}-resistant lines (**Figures 4.12d and 5.12d**). This indicates that the roots of plants Al^{3+}-stressed plants in acid soil become short, thickened and fewer in number, as previous reported by Foy (1984). Loss of thin roots will reduce the contact areas among roots and soil, and further decrease the uptake of nutrient and water in soil. All of the lines show good rhizosheath in limed soil, while loss of rhizosheath in acid soil, especially for the parental line Golden Promise and Null lines (**Figures 3.11**, **4.6** and **5.6**), which indicates small root hairs (Delhaize *et al.*, 2012).

The question of why organic anions efflux is such a widespread mechanism of Al^{3+} resistance in plants has been discussed elsewhere. First, malate and citrate are very economic small organic compound, and the stability of the [Al^{3+}:malate] and [Al^{3+}:citrate] complexes is critical to their role in protecting plants from Al^{3+} toxicity. Second, these organic acids are ubiquitous in living cells and metabolically cheap to synthesis. They participate in key anabolic and catabolic pathways including the tricarboxylic acid cycle, the glyoxylate cycle, C_4 photosynthetic carbon reduction and crassulacean acid metabolism. Ryan et al.(2010) proposed that

the evolution of Al^{3+} resistance via organic anion efflux arose from mutations that co-opted citrate and malate transport proteins from other functions. Mutations that cause the misdirection of these transport proteins from one cell type to another or from one membrane to another membrane may induce it to perform a new function like releasing organic anion efflux from roots, which provides a degree of Al^{3+} resistance. In the process of evolution, those mutations in MATE genes may confer Al^{3+}-resistance instead of iron translocation by altering their expression tissues (Magalhaes, 2010). For example, the adaptation of barley to acid soil was achieved by modification of a single citrate transporter gene *HvAACT1*. The primary function of this gene is to release citrate from root pericycle cells to xylem to facilitate the translocation of iron from root to shoot. However, 1 kb insertion in the upstream of *HvAACT1* coding region is only found in Al^{3+} resistant accessions. This mutation enhances the expression of *HvAACT1* and extends the expression location to the root tips (Fujii *et al.*, 2012). The primary function of *Frd3* is to assist the translocating iron from root to shoot in *Arabidopsis* by releasing citrate into vascular tissue (Green and Rogers, 2004). The citrate is thought to chelate the Fe(II) and Fe(III) and move up the xylem as a complex, but when it was over-expressed in *Arabidopsis* with a constitutive promoter the citrate efflux from root apices increase Al^{3+} resistance (Durrett et al., 2007). Like *Frd3* in *Arabidopsis*, when barley *HvALMT1* gene was over-expressed in Al^{3+}-sensitive barley cultivar Golden Promise, it conferred Al^{3+}-independent malate efflux and an increase in Al^{3+} resistance, while none of barley germplasm in nature show malate efflux from root apices (Gruber et al., 2011). This is very meaningful to investigate the evolution of organic transporters that confer Al^{3+} resistance.

The results in this study also highlight the importance of including null segregant lines as controls in transgenic experiments rather than just parental lines. For instance, there were significant differences in relative root fresh weight, relative longest root growth and relative second longest root length between the wild-type Golden Promise and null lines even though these should be genetically identical (**Figure 4.8**). The differences have to be caused by either the growth conditions used to generate the seed (affecting the nutrition of the seed etc) or somaclonal variation generated during the tissue culture phase (Larkin and Scowcroft, 1981).

In natural barley populations, a positive of correlation was observed between the amount of citrate efflux and relative root growth in 21 barley germplasm (Zhao et al., 2003). However in the transgenic plants generated in this study, a correlation was not found between citrate efflux and Al^{3+} resistance and this was unexpected. It is likely that a higher number of individual transgenic lines is required to detect such an observation.

Additional increases in the Al^{3+} resistance of barley could be achieved by combining this transgenic approach with other strategies. For instance the natural resistance of Dayton and other resistance cultivars can be combined with the transgenes tested here as well as others (eg. *TaALMT1*) to see if resistance can be increased. It may be possible to obtain additive effects by combining the MATE (citrate efflux) and ALMT (malate efflux) transporters in the same plant. Lines can be crossed to ALMT1-barley, and their F1 seeds will be grown to harvest F2 seeds. F3 seeds from individual F2 seed are screened for *MATE* and *TaALMT1*, and the homozygous lines containing these two genes will be obtained. These lines can be used to cross with other transgenic MATE lines. Insert copy number is an important consideration if these transgenic lines are used in crosses to increase the Al^{3+} resistance of other elite cultivars. Multiple inserts makes introgression and transfer of a full phenotype into another plant more difficult. During the process, Al^{3+}-resistant genes should be pyramid one by one.

Alternatively enhancing the expression of other genes which control the synthesis of citrate could be beneficial – especially if citrate supply limits citrate efflux. There are indications that this is the case because regardless of the *MATE* used in this study the transgenic lines were never more resistant than the wild-type cv. Dayton. If the supply of organic anions becomes limiting for efflux, genes involved in increasing organic anion biosynthesis could be expressed together with organic anion transporters, to generate transgenic lines with higher rate at citrate efflux and greater Al^{3+}resistance. There are reports of enhanced Al^{3+} resistance when citrate synthesis (CS) expression was increased in alfalfa (Barone et al., 2008), *Arabidopsis* (Koyama et al., 2000, Koyama et al., 1999) and tobacco (Han et al., 2009). However Delhaize et al.(2001) found that transgenic tobacco plants with CS from *Pseudomonas aeruginosa* did not exhibit enhanced either citrate accumulation

or efflux. So the efficiency of this strategy remains uncertain. Additional benefits may also be gained by transforming other Al^{3+}-resistance related genes into these transgenic lines. Previous studies have increased Al^{3+} resistance of plants by over-expression genes responsible for membrane physiology and stress responses (Basu et al., 2001, da Silva *et al.*, 2006, Ezaki et al., 2000, Ryan et al., 2007) or genes involved in organic anion metabolism (Trejo-Tellez *et al.*, 2010).

With the increasing food requirement all over the world, raising the productivity of marginal land is essential. Careful land management and Al^{3+}-resistant germplasm are complementary strategies for managing acid soils. Agronomic solutions involving the application of lime can be too costly for farmers in poor countries because of up-front cost required for transportation and distribution of lime. Transgenic strategies provide the potential of allowing normally acid-sensitive crops such as barley to be grown effectively on a wide range of acid soils.

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Appendix

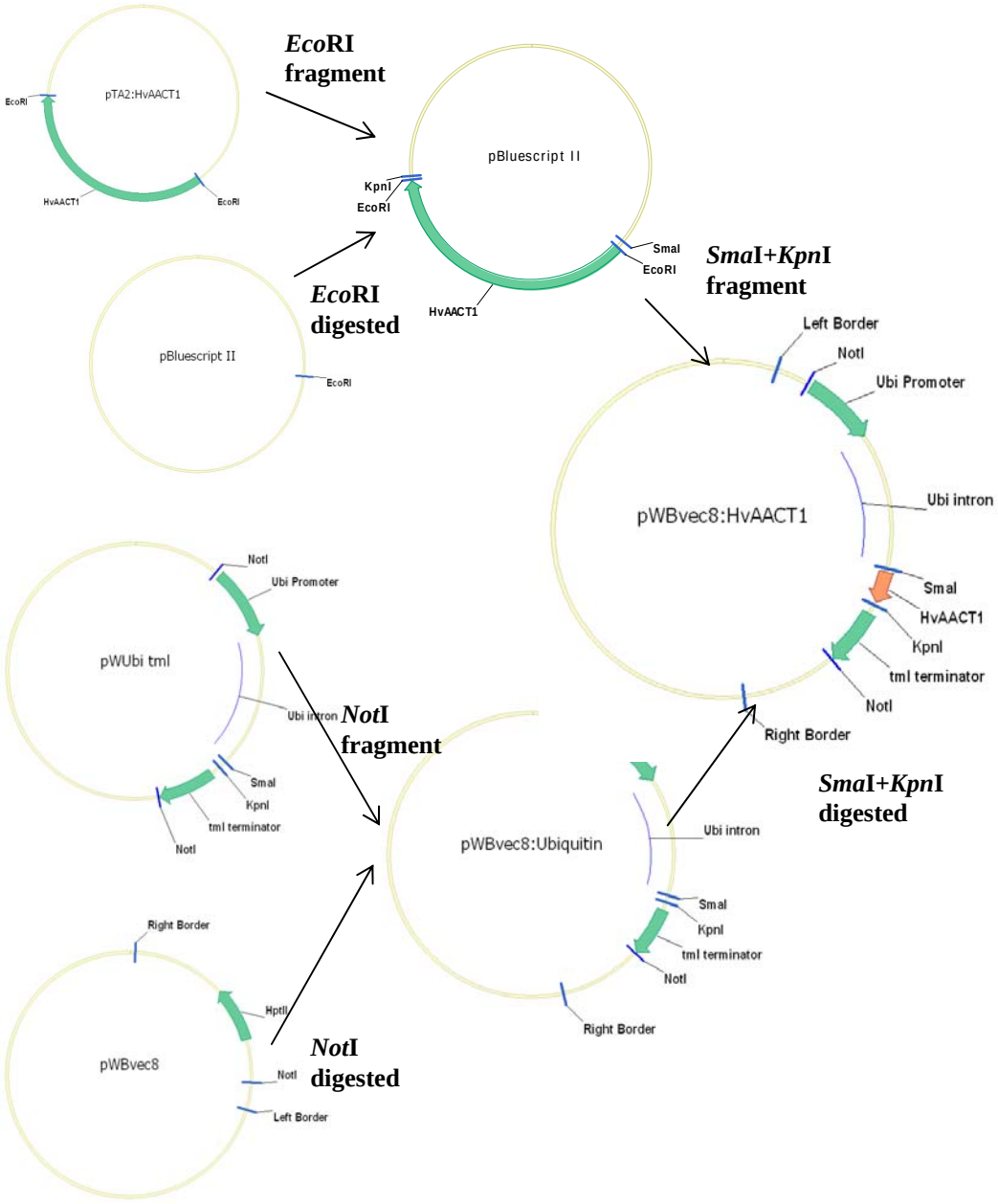

Figure S1 Construction of pWBvec 8:HvAACT1 vector for transformation

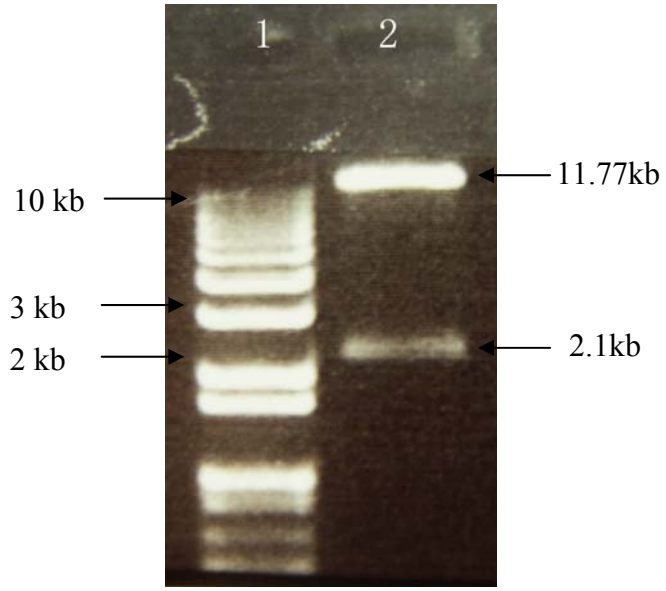

Figure S2 pWBVec8:ubiquitin digested with *Bam*HI

1：1kb^{+}Marker
2： pWBvec8:ubiquitin

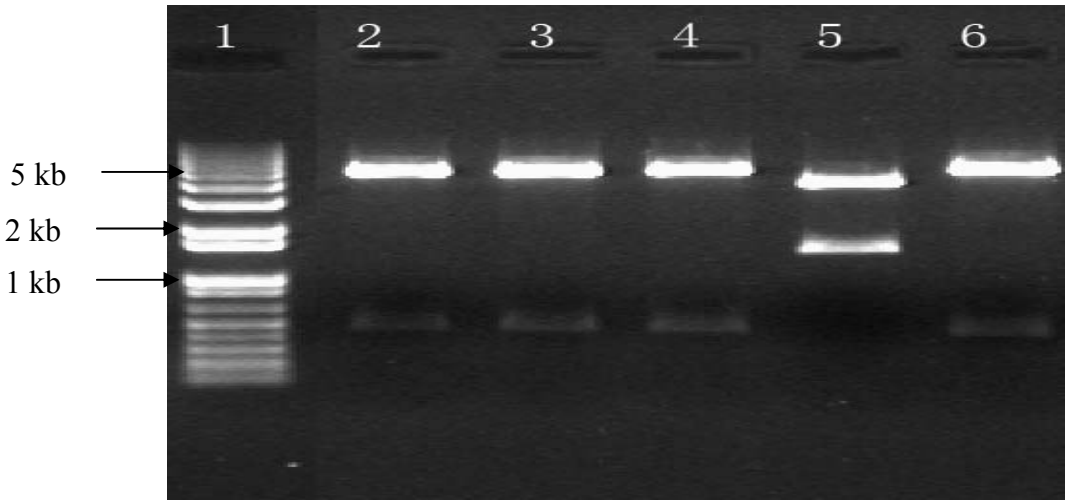

Figure S3 pBluescript II:HvAACT1 digested with *Pst*I

The correct fragment is shown in lane 5 (1.3 kb and 3.4 kb).

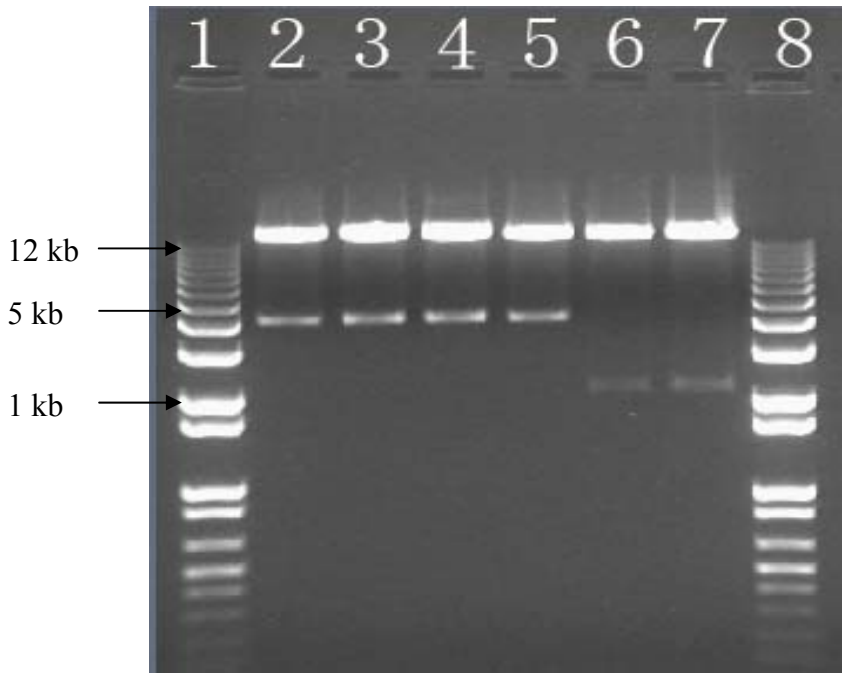

Figure S4 *pWBvec8:HvAACT1* double digested with *Sma*I and *Kpn*I

1,8：1Kb+Marker
2-5：*pWBvec8:HvAACT1*
6,7：*pWBvec8:ubiquitin* (No insert)

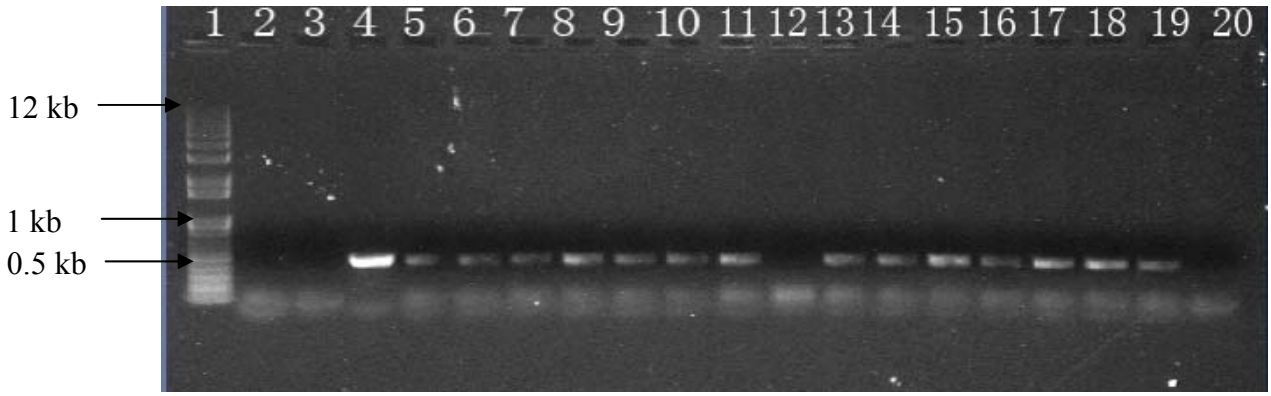

Figure S5 Identification of *HvAACT1* transgenic barley by PCR

1：1Kb+Marker
2： H_2O
3：GP
4： plasmid *pWBvec8:HvAACT1*
5-20： T0 *HvAACT1* barley

Table S1 Details of elemental analysis of barley grown in iron deficient and sufficient solutions. Germinated barley seed (Wild-type cv. Golden Promise, Null line Null_Frd3_40 and two *Frd3* T3 lines 40 and 55) were grown in hydroponic solution containing 5 µM (deficient) and 100 µM (sufficient) Fe^{3+} for 15 days. Roots and shoots were harvested and dried and then conducted elemental analysis for one or two replicates.

Treatment	Tissue	Lines	Al	B	Ca	Cr	Cu	Fe	K	Mg	Mn	Na	P	Pb	S	Ti	Zn
			mg/kg	mg/kg	%	mg/kg	mg/kg	mg/kg	%	%	mg/kg	%	%	mg/kg	%	mg/kg	mg/kg
Low iron	Shoot	Golden Promise	22.8	14.8	0.617	0.39	27.8	33.4	6.93	0.329	291	0.0287	1.09	1	0.845	0.77	255
Low iron	Shoot	Golden Promise	36.6	17.4	0.823	0.36	46.5	40.2	7.01	0.348	246	0.0135	1.08	1.4	1.04	1.4	220
Low iron	Shoot	Null_Frd3_40	19.6	7.96	0.48	0.68	29.6	32.7	6.83	0.226	212	0.0169	1.15	0.5	0.757	0.56	292
Low iron	Shoot	Null_Frd3_40	21	16.9	0.578	0.32	30.1	29.6	6.36	0.330	261	0.0142	0.944	0.7	0.82	0.61	263
Low iron	Shoot	T3_Frd3_40	21.8	20.6	0.410	0.3	29.9	27.7	6.46	0.328	269	0.0051	1.16	0.9	0.863	0.61	224
Low iron	Shoot	T3_Frd3_40	26.5	29.4	0.503	0.28	42.3	38.9	6.61	0.299	193	0.0194	0.985	0.7	0.848	0.47	175
Low iron	Shoot	T3_Frd3_55	35.1	21.3	0.404	0.35	39.1	36.4	6.98	0.220	215	0.0149	0.939	0.2	0.9	0.61	157
Low iron	Shoot	T3_Frd3_55	30.3	14.2	0.430	0.3	37.0	39.7	6.31	0.233	251	0.0091	0.980	1	0.919	0.51	192
Low iron	Root	Golden Promise	99.3	8.94	0.233	0.77	1310	64.1	4.45	0.0768	138	0.0405	0.858	4.5	0.729	1.4	65.5
Low iron	Root	Golden Promise	98	8.2	0.224	0.68	1660	65.3	4.91	0.0743	143	0.0405	0.880	4.3	0.783	1.1	65.3
Low iron	Root	Null_Frd3_40	97.9	9.12	0.240	0.64	1560	66.4	4.95	0.0799	156	0.0432	0.917	4.3	0.792	1.9	66.1
Low iron	Root	Null_Frd3_40	106	9.93	0.280	0.62	1580	63.4	5.19	0.0839	177	0.0426	0.894	4	0.836	1.3	65.1
Low iron	Root	T3_Frd3_40	75.4	9.28	0.217	0.88	1170	63.9	4.31	0.0753	150	0.048	0.840	3.5	0.714	1.3	57.1
Low iron	Root	T3_Frd3_55	81.3	7.56	0.215	0.66	2480	66.1	5.28	0.0691	177	0.0413	1.01	3.8	0.99	1.3	72.6

Table S1 Continued

Treatment	Tissue	Lines	Al	B	Ca	Cr	Cu	Fe	K	Mg	Mn	Na	P	Pb	S	Ti	Zn
			mg/kg	mg/kg	%	mg/kg	mg/kg	mg/kg	%	%	mg/kg	%	%	mg/kg	%	mg/kg	mg/kg
Low iron	Root	T3_Frd3_55	84.3	7.45	0.211	1.66	2090	71.7	5.42	0.0711	172	0.0396	1.11	4.1	0.89	1.2	67.0
High iron	Shoot	Golden Promise	26.1	15.9	0.659	0.19	27.0	63.5	6.71	0.257	130	0.0189	0.873	0.8	0.563	0.22	85.8
High iron	Shoot	Golden Promise	26.9	19.5	0.742	0.29	23.6	76.1	7.44	0.281	122	0.0227	0.914	0.5	0.489	0.49	63.7
High iron	Shoot	Null_Frd3_40	26.8	11.7	0.676	0.28	23.3	67.3	7.26	0.223	103	0.0323	0.992	0.3	0.401	0.54	89.6
High iron	Shoot	Null_Frd3_40	22.3	14.4	0.703	0.17	23.1	66.6	7.11	0.247	101	0.0282	1.02	0.5	0.48	0.4	99.4
High iron	Shoot	T3_Frd3_40	21.9	18.6	0.589	0.13	27.6	68.6	7.10	0.255	124	0.0181	0.792	0.4	0.487	1.6	96
High iron	Shoot	T3_Frd3_40	22.9	14	0.600	0.14	26.6	71.3	7.34	0.234	133	0.013	0.832	0.2	0.584	1.2	83.1
High iron	Shoot	T3_Frd3_55	24.4	15.8	0.748	0.29	31.0	108	7.65	0.262	131	0.0268	0.813	0.9	0.624	0.98	84.0
High iron	Shoot	T3_Frd3_55	24.9	23.8	0.636	0.15	25.6	76.2	7.34	0.202	109	0.0133	0.828	0	0.569	1.2	84.3
High iron	Root	Golden Promise	62.7	8.76	0.202	0.72	410	1180	5.43	0.0664	257	0.099	0.835	25	0.382	1.6	72.8
High iron	Root	Null_Frd3_40	86.9	10.6	0.233	0.86	449	1340	6.20	0.0714	330	0.0887	0.903	28	0.482	1.8	89.4
High iron	Root	T3_Frd3_40	72.5	10.1	0.256	1.36	715	972	6.30	0.0655	363	0.0603	0.905	21	0.478	1.7	97.2
High iron	Root	T3_Frd3_55	85.9	9.49	0.239	1.39	690	1160	6.33	0.0643	368	0.0692	0.847	25	0.513	1.8	103

Table S2 Elemental analysis of seed from homozygous transgenic *ALMT1* and *MATE* barley lines. These plants were grown in pots filled with normal soil in glasshouse. Their seed were harvested for elemental analysis using the method described in Chapter 5. 'Golden Promise', Al^{3+}-sensitive parental cultivar; 'Dayton', Al^{3+}-resistant barley cultivar; 'TaALMT1-barley', transgenic *TaALMT1* barley line; 'Null_', nontransgenic lines; 'HvAACT1_', transgenic *HvAACT1* barley lines; 'SbMATE_', transgenic *SbMAT*E barley lines; 'Frd3_', transgenic *Frd3* barley lines.

	Al	B	Ca	Cu	Fe	K	Mg	Mn	Na	P	S	Zn
	mg/kg	mg/kg	%	mg/kg	mg/kg	%	%	mg/kg	%	%	%	mg/kg
Golden Promise	7	3.3	0.030	4.6	31	0.503	0.114	13.7	0.0033	0.346	0.091	23.1
Dayton	3	1.9	0.020	5.0	20	0.477	0.121	11.6	0.0012	0.342	0.086	25.8
TaALMT1-barley	2	2.8	0.029	4.8	31	0.575	0.136	15.2	0.0024	0.402	0.137	35.3
Null_HvAACT1_ 17A	1	9.5	0.030	5.7	20	0.451	0.148	12.6	0.0014	0.387	0.108	33.6
Null_HvAACT1_ 33A	3	6.1	0.031	7.2	35	0.546	0.147	14.9	<0.0003	0.407	0.124	30.8
Null_HvAACT1_51	1	8.7	0.029	7.0	42	0.460	0.163	15.6	0.0012	0.433	0.146	42.1
HvAACT1_ T2_17A	2	8.4	0.031	5.4	23	0.511	0.141	12.4	0.0020	0.373	0.098	33.8
HvAACT1_ T2_33A	1	6.3	0.037	5.6	25	0.501	0.126	12.6	0.0019	0.349	0.094	23.4
HvAACT1_ T2_51	1	6.7	0.043	8.2	48	0.509	0.167	18.7	0.0013	0.480	0.155	52.4
HvAACT1_ T2_52B	1	10.1	0.033	8.7	47	0.580	0.160	17.7	0.0025	0.465	0.157	46.8
Null_SBMATE_100E	1	0.3	0.024	5.9	20	0.515	0.135	11.0	0.0020	0.384	0.104	27.3
Null_SbMATE_22	1	3.9	0.016	7.1	52	0.771	0.115	11.9	0.0028	0.464	0.133	45.6
SbMATE_ T3_9A	1	0.6	0.026	5.8	27	0.561	0.139	14.4	0.0016	0.393	0.120	34.3
SbMATE_ T3_22	1	0.5	0.031	6.3	22	0.504	0.134	14.0	0.0021	0.404	0.117	26.3
SbMATE_ T3_100E	1	0.4	0.028	7.1	25	0.527	0.133	13.0	0.0027	0.413	0.129	28.5
SbMATE_ T3_133	1	0.3	0.024	6.1	29	0.588	0.124	10.9	0.0032	0.379	0.108	28.5
Null_Frd3_40	1	5.7	0.029	7.3	36	0.498	0.153	17.8	0.0011	0.412	0.136	45.6
Null_Frd3_55	1	2.7	0.029	6.4	42	0.525	0.147	14.7	0.0028	0.411	0.120	46.2
Frd3_ T3_40	2	9.5	0.037	7.0	29	0.505	0.144	17.0	0.0013	0.404	0.123	40.5
Frd3_ T3_55	1	4.1	0.036	6.2	29	0.502	0.146	15.9	0.0025	0.395	0.113	35.5